The Phospholipase C Signalling System: Study of the role of the G protein and identification of a novel variant of Phospholipase C 4

Thesis

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THE PHOSPHOLIPASE C SIGNALLING SYSTEM

Study of the role of the G_O protein and identification of a novel variant of Phospholipase C β4

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DECLARATION

This thesis has been written by myself and has not been used in any other previous application for a degree.

The work on Ltk^- clones presented in the thesis has been mainly performed by Micaela Grandolfo with my help. The isolation of Plc β4 c cDNA from a rat brain library and the reconstruction of the full-length sequences of Plc β4 a and Plc β4 c have been performed by Barbara Marinelli. All other results were obtained by myself. Part of the data contained in this thesis has been presented at ECBO 1999, Bologna, Italy (L. De Filippis, B. Marinelli, C. Muca and L. Vallar; Identification and characterization of an ubiquitously expressed variant of phopsholipase C β4; Abstract A65). All sources of information are acknowledged by means of reference.

ABSTRACT

The thesis focuses on the properties and regulation of phospholipase C β (PLC β). Studies concerning the effect of $G\alpha_o$ subunit on PLC activity revealed that mutationally activated $G\alpha_o$ inhibits PLC activity in mouse Ltk^- clones. The observed effect seemed to be specific of $G\alpha_o$, as either wild-type $G\alpha_o$ or the wild-type and mutationally activated $G\alpha_{i1}$ subunits were not able to affect receptor-mediated activation of PLC. Known second messengers as Ca^{2+} and cAMP were not involved in this inhibitory process since they were not able to mimic or prevent the $G\alpha_o$ effect on PLC. Transient transfection experiments in COS7 cells did not riproduce the same results; therefore, either a direct or an indirect mechanism of PLC activity regulation by $G\alpha_o$ remain feasible hypothesis. The thesis also presents results concerning the identification of a novel C-terminal variant of PLC β4 (PLC β4c), that has a 37-nucleotide insertion which results in replacement of 22 aminoacid residues at the carboxyl terminal tail of PLC β4a with 41 unique residues. RT-PCR and Western Blot experiments did show that, in contrast with the known enzyme (PLC β4a) detectable only in brain, PLC β4c is present in several tissues and cell lines. Expression of full-length cDNAs in COS7 cells revealed that the two forms of PLC β are associated with the particulate fraction and equally responsive to G protein subunits. However, the different

carboxyl terminal tails of PLC β4 isoforms may allow for differential targeting and subcellular localization, contributing to regulation of PLC β4 - mediated specific pathways. Even the presence of a PDZ domain binding sequence in PLC β4a but not in PLC β4c suggests functional differences between the two variants.

ABBREVIATIONS

AC= adenylate cyclase

ACPD=1-amino-1,3-cyclopentanedicarboxylic acid

ADP=adenosine diphosphate

AR=adrenergic receptor

ATP= adenosine triphosphate

βArk= β adrenergic receptor kinase

bp= base pairs

BCA= bicinchonic acid

BSA= bovine serum albumine

Btk= Bruton's tyrosine kinase

cAMP= cyclic adenosine monophosphate

Cch= carbachol

cDNA= complementary DNA

CHO= Chinese Hamster Ovary (cells)

Csk= C-terminal src kinase

CTX= Cholera toxin

DMEM= Dulbecco's modified Eagle's medium

DNA= desossiribonucleic acid

ECL= enhanced chemioluminescence

EGF=epidermal growth factor

ERK= extracellular signal-regulated kinase

FGF= fibroblast growth factor

g= gravity

GAP=GTPase activating protein

GEF= guanine nucleotide exchange factor

GH= growth hormon

GIRK= G protein-coupled inwardly rectifying K^+ channel

GOA= *C.elegans* G_o α subunit

GDP= guanosine diphosphate

GPCR= G protein coupled receptor

Grb=growth factor receptor binding protein

GTP= guanosine triphosphate

HL60= herytroleukemia (cell line)

INAD= inactivation no-after potential

IP= inositol phosphate

IRS= insulin receptor substrate

KLH= keyhole limpet hemocyanin

LPA= lysophosphatidic acid

LT= leukotrien

LTD= long term depression

mAChR= muscarinic acetylcholine receptor

MAPK= mitogen-activated protein kinase

mGluR= metabotropic glutamate receptor

MCPP= 1-(3-clorophenyl) piperazine

NGF= nerve growth factor

NORPA= no receptor potential A

PAF= platelet-activated factor

PC12= pheochromocitoma 12(cell line)

PCR= polymerase chain reaction

PDGF= platelet derived growth factor

PDZ= post-synaptic density 95, disc-large and zonulin-1 protein (domain)

PGE= prostaglandin E

PH=pleckstrin homologous (domain)

PI3= phosphatydilinositol-3 phosphate

PI4K= phosphatydilinositol-4 phosphate kinase

PIP2= phosphatydil-inositol 4,5 bisphosphate

PKA= protein kinase A

PKC= protein kinase C

PLA2= phospholipase A2

PLC= phospholipase C

PLD= phospholipase D

PMA= phorbol myristate acetate

PTX= pertussis toxin

Raf= ras activating factor

RGS= regulator of G protein signaling

RNA= ribonucleic acid

RT= reverse transcriptase

SDS= sodium dodecyl sulphate

SE= standard error

Shc= src homolog and collagen

Stat3= signal transducer and activator of transcription

SV40= Simian Virus 40

TG= transglutaminase

TRH= thyroid releasing hormon

TRP= transient receptor potential (channel)

TSH= thyroid stimulating hormon

TXA= thromboxane

UTR= untranslated region

INTRODUCTION

CELL SIGNALLING

Each cell in a multicellular animal is programmed during development to respond to a specific set of signals that act in various combinations to regulate its behavior, beginning with life or death, with proliferation or quiescence. Intercellular signals, interpreted by complex machinery in the responding cell, allow each cell to determine its position and specialized role in the body and ensure, for example, that each cell divides only when its neighbors dictate that it should do so. The importance of such "social controls" on cell division becomes apparent when the controls fail, resulting in cancer, which often kills the multicellular organism.

An animal cell contains an elaborate network of proteins that enables the cell to respond to signals from other cells. The system includes cell-surface and intracellular receptor proteins, protein kinases, protein phosphatases, GTP-binding proteins, and the many intracellular proteins with which these signalling proteins interact.

Mechanisms enabling one cell to influence the behavior of another almost certainly existed in the world of unicellular organisms long before multicellular organisms appeared on earth. Evidence comes from studies of some present-day unicellular eucaryotes such as yeasts. Although these cells normally lead independent lives, they can communicate and influence one another's proliferation in preparation for sexual mating (Marsh et al., 1991).

In the budding yeast *Saccharomyces cerevisiae*, for example, when a haploid individual is ready to mate, it secretes a peptide mating factor that signals cells of opposite mating types to stop proliferating and prepare to conjugate; the subsequent fusion of two haploid cells of the opposite mating types produces a diploid cell, which can then undergo meiosis and sporulate to generate haploid cells with new assortments of genes. Studies of yeast mutants that are unable to mate have identified many proteins that are required in the signalling process. These proteins form a signalling network that includes cell-surface receptors, GTP-binding proteins, and protein kinases, each of which has close relatives among the proteins involved in signalling in animal cells. Through gene duplication and divergence, however, the signalling systems in animals have become much more elaborate than those in yeasts.

Whereas yeast cells communicate with one another for mating by secreting several kinds of small peptides, cells in higher organisms communicate by means of hundreds of signalling molecules, including proteins, small peptides, amino acids, nucleotides, steroids, retinoids, fatty acid derivatives, and even dissolved gases such as nitric oxide and carbon monoxide. Most of these signalling molecules are secreted from the signalling cell by exocytosis. Others are released by diffusion through the plasma membrane, while some remain tightly bound to the cell surface and influence only cells that contact the signalling cell.

Regardless of the nature of the signal, the target cell responds by means of a specific protein called a receptor. It specifically binds the signalling molecule and initiates a response in the target cell. Many of the extracellular signalling molecules act at very low concentrations, and the receptors that recognize them usually bind them with high affinity. In most cases the receptors are transmembrane proteins on the target-cell surface. When they bind an extracellular signalling molecule (a ligand), they become activated so as to generate a cascade of intracellular signals that alter the behavior of the cell. In some cases, however, the receptors are inside the target cell and the signalling ligand has to enter the cell to activate them: these signalling molecules therefore must be sufficiently small and hydrophobic to diffuse across the plasma membrane.

There are three main families of cell-surface receptors, each of which transduces extracellular signals in a different way. Ion-channel receptors are transmitter-gated ion channels, involved in rapid synaptic signalling between electrically excitable cells. This type of signalling is mediated by a small number of neurotransmitters that increases transiently the probability for the channel to be open or closed. G-protein-linked receptors activate or inactivate plasma-membrane-bound enzymes or ion channels via trimeric GTP-binding proteins (G proteins). Enzyme-linked receptors either act directly as enzymes or are associated with enzymes; the latter are usually protein kinases or phosphatases that phosphorylate or dephosphorylate

specific proteins of the target cell. Through cascades of highly regulated protein phosphorylations, elaborate sets of interacting proteins relay most signals from the cell surface to the cytoplasm or to the nucleus, thereby altering the cell's metabolism or the cell's pattern of gene expression and, as a consequence, its behavior. Other signals are addressed to the regulation of cellular metabolic processes, such as glycogen breakdown, due to the activation of cyclic-AMP-dependent protein kinase (A-kinase) (Cohen, 1983). Cross-talk between different signalling cascades enables a cell to integrate information from the multiple signals that it receives.

G PROTEINS

A large number of neurotransmitters, hormones and other extracellular ligands, but also light and odorants, elicit cellular responses by interaction with one receptor of the largest family, those coupled to G proteins. These receptors are cell membrane proteins characterized by seven hydrophobic membrane-spanning domains (Baldwin, 1994). They are coupled by heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) to intracellular effectors, either enzymes or ion channels, that generate intracellular signals leading to cellular responses. G protein-mediated signalling pathways have attracted a great deal of attention because of the

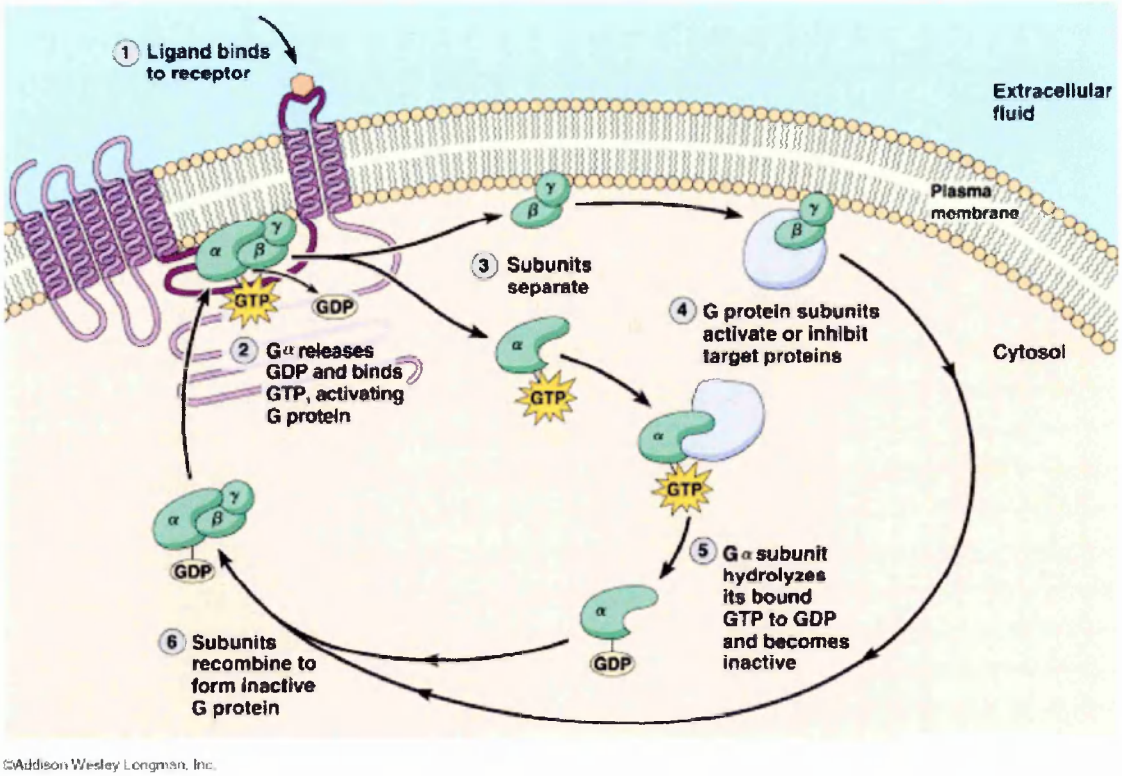


Fig.1 The G protein cycle. G protein-linked receptors contain seven transmembrane segments that form a ligand-binding site on the outside of the cell and a G protein-binding site on the inside. (1) When the ligand binds, (2) the receptor activates a G protein by causing the Ga subunit to release GDP and acquire GTP. (3) The G(α) and G(βγ) subunits then separate and (4) initiate signal transduction events. (5) The GTP-G(α) subunit eventually hydrolyzes its bound GTP, converting the subunit back to its inactive GDP-G(α) form. (6) The inactive GDP-G(α) subunit then recombines with G(βγ) to form the inactive G heterotrimer.

many physiological and pharmacological processes modulated or mediated by these mechanisms.

Heterotrimeric G proteins consist of an α subunit that binds and hydrolyzes GTP together with a β and a γ subunit (Casey and Gilman, 1988; Birnbaumer 1990; Bourne et al., 1990; Simon et al., 1991; Spiegel 1992; Neer 1995; Hamm 1998). It is the identity of the α subunit that defines the heterotrimer. The β and γ subunits are tightly bound to each other and are regarded as a functional entity, the βγ complex. To transmit a signal from the receptor to effectors, the heterotrimeric G protein undergoes an activation-inactivation cycle, which allows it to function as a molecular switch. In the inactivated state, the α subunit is bound to GDP and is tightly associated with the βγ complex (Fig.1). In this form, the G protein can be recognized by an appropriate receptor that has been activated by a specific stimulus. The activated receptor binds to the heterotrimer and promotes the dissociation of GDP from the α subunit. GDP is then replaced by GTP, whose intracellular concentration exceeds that of GDP by about 10 fold. The accepted model for activation of the heterotrimer states that binding of GTP induces a conformational change in the α subunit that results in the dissociation of the protein from receptor as well as from βγ complex. Both α-GTP and the βγ complex are able to interact with effector proteins and regulate their function. Activation is terminated by the intrinsic GTPase activity of the α subunit,

which hydrolyses GTP. GDP-bound α reassociates with the free βγ complex and the heterotrimer is ready to enter a new cycle. Recent studies have reexamined this hypothesis, by using fusion proteins between the yeast *Saccharomyces cerevisiae* G β subunit (Ste4) and G α subunit (Gpa1) (Klein et al., 2000) or between the α factor-receptor (Ste2) and Gpa1 (Medici et al., 1997): both the chimeric proteins allow efficient signal transduction as effectively as when coexpressed as separate proteins. Hence, at least in yeast, dissociation of the heterotrimer by the receptor or dissociation of the α subunit by the βγ complex is not required for the activation of the α subunit.

Some effectors, such as phospholipase Cβ and the retinal cGMP phosphodiesterase appear able to accelerate hydrolysis of GTP by the α subunit (Berstein et al., 1992; Biddlecome et al., 1996; Arshavsky and Bownds, 1992). In addition, the recently identified proteins called regulators of G protein signalling (RGS) interact with GTP-bound α subunits and stimulate their GTPase activity (Dohlman and Thorner, 1997; Arshavsky and Pugh, 1998; Berman and Gilman, 1998; Zerangue and Jan, 1998).Thus, RGS family members are thought to govern the strength and duration of physiological responses triggered by G protein-dependent signalling pathways.

G protein α subunits

Heterotrimeric G proteins are a subset of a larger family of GTP-binding proteins. This larger family includes monomeric factors that control protein synthesis and a group of monomeric GTP-binding proteins, such as Ras, Rab and Rho, involved in the regulation of proliferation, intracellular transport, and other cell functions (Bourne et al., 1990; Kaziro et al., 1991). It is the α subunit of heterotrimeric G proteins that has structural and functional homologies with other GTP-binding proteins.

The elucidation of the crystal structure of GDP and GTP-bound transducin and α_{i1} has provided a picture of the molecular mechanism of guanine nucleotide binding and hydrolysis by G protein α subunits (Neer, 1995). Basically, X-ray crystallographic analysis has revealed that Gα subunits contain two domains: a domain involved in guanine nucleotide binding and GTP hydrolysis, which is homologous to other GTP-binding proteins, and a unique helical domain (Bourne et al., 1991; Spiegel et al., 1992; Hamm and Gilchrist, 1996). The GTPase domain contains the sites for binding βγ subunits, receptors and downstream effectors. The helical domain seems to contribute to the stability of the guanine nucleotide binding site (Hamm, 1998). However, its main function is not yet known.

The C-terminal part of the α subunit is involved in interaction with receptors. In particular, it has been shown that the last few residues of the protein

contribute importantly to the specificity of receptor-G protein interaction (Conklin and Bourne, 1993; Bourne 1997; Hamm and Gilchrist, 1996). Mutational analysis and peptide mapping studies have narrowed down important interaction sites of α_s, α_t and α_q with their respective effectors (adenylyl cyclase, cGMP phosphodiesterase and phospholipase Cβ) to regions in the C-terminal half of the α subunit (Berlot and Bourne, 1992; Rarick et al., 1992; Arkinstall et al., 1995). Finally, the N-terminus of the α subunit and the effector binding site are important for interaction with the βγ complex (Conklin and Bourne, 1993; Rens-Domiano and Hamm, 1995).

More than twenty different mammalian G protein α subunits have been described so far. On the basis of their sequence homology and common functional properties, they can be divided into four classes (Fig.2):

1) **Gα_s**: this class includes α_s, that couples a large group of membrane receptors (such as the β-adrenergic receptor and the TSH receptor) to adenylyl cyclase, and α_{olf}, that couples odorant receptors to adenylyl cyclase. In addition to the stimulation of adenylyl cyclase, it has been reported that Gα_s also activates dihydropyridine-sensitive Ca^{2+} channels in skeletal and cardiac muscle (Brown and Birnbaumer, 1988). Most tissues contain a mixture of two major forms of G α_s that migrate with apparent molecular masses of 45 and 52 kDa. This heterogeneity arises from alternative splicing

Class	Subunits	Effectors and effects
α_s	α_s, α_{olf}	Stimulation of adenylyl cyclase
$\alpha_{i/o}$	α_{i1}, α_{i2}, α_{i3}	Inhibition of adenylyl cyclase Activation of K^+ channels
	α_o	Inhibition of adenylyl cyclase Regulation of B-Raf, Rap1GAP, the Stat3 and MAPK pathways
	α_z	Inhibition of adenylyl cyclase
	α_t	Stimulation of cGMP phosphodiesterase
	α_{gust}	Regulation of bitter and sweet signalling pathway
α_q	α_q, α_{11}, α_{14}, $\alpha_{15/16}$	Stimulation of phospholipase C β Activation of tyrosine kinase Btk
$\alpha_{12/13}$	α_{12}, α_{13}	Activation of the GTPase CDC42, Rho, Rac Inhibition/stimulation of p115 RhoGEF Activation of Ras GAP Activation of the tyrosine kinase Btk

Fig.2. G protein α subunits and some of their functions.

of a single precursor mRNA, which results in polypeptides that contain (referred to as long form) or lack (short form) a sequence of 25 residues encoded by exon III of the gene for G α_s. In fact, as many as four splice variants appear to be produced by this mechanism, as each short and long form may include or exclude a single serine residue at the splice junction (Kozasa et al., 1988).

G α_s is sensitive to cholera toxin (CTX) that is able to catalyze ADP-ribosylation of an arginine residue (Arg201 in the long form of α_s). This modification affects the α chain from two connected aspects: inhibition of the GTPase activity and persistent activation by the bound GTP. As a consequence, G α_s raises intracellular cAMP levels independently of the presence of extracellular signals. It is commonly known that the severe watery diarrhea characteristic of clinical infection with *Vibrio cholerae* is secondary to the direct effect of CTX on G α_s which induces the activation of Cl^- channels in intestinal epithelial cells, although Peterson and Ochoa (1989) analyzed the role of cAMP and prostaglandins in the pathogenesis of experimental cholera and demonstrated that cAMP accumulation triggers only a small, transient intestinal fluid accumulation response, while prostaglandin E is responsible for long-term water and electrolyte secretion in cholera.

Mutations in the gene encoding G α_s are involved in the development of various human diseases (Spiegel et al., 1992). Interestingly, analysis of DNA

from human GH-secreting pituitary tumors and thyroid tumors revealed somatic mutations within the G α_s gene that are single substitutions of residue Gln227 or residue Arg201 (the site of covalent modification by CTX). These substitutions inhibit the intrinsic GTPase activity of G α_s and, in cells in which cAMP stimulates proliferation, lead to abnormal cell growth (Spiegel et al., 1992; Spiegel et al., 1993; Spiegel, 1995; Vallar, 1996). The mutant activated α_s genes found in human tumors have been termed gsp (G_s protein) oncogenes (Landis et al., 1989).

2) **G** $\alpha_{i/o}$. This group includes several types of α subunits: α_i, α_o, α_z, α_t and α_{gust}. Although closely related, the α_i subunits, α_{i1}, α_{i2} and α_{i3}, are encoded by different genes (Freissmuth et al., 1989). Several studies have shown that it is possible to assign the sequence encoded by the α_{i1} gene to a 41-kDa protein found in high concentrations in the brain, the α_{i2} gene to a 40-kDa protein that appears to be relatively ubiquitously distributed, and the α_{i3} gene to a distinct 41-kDa protein whose distribution is prevalent in non neural tissues. The proteins referred as Gα_o and Gα_z are mainly expressed in the brain. The effectors and pathways regulated by the α_i subunits have been at least partially characterized (Neer, 1995; Hamm, 1998), whereas the signalling functions of Gα_o and Gα_z are less clear. The two isoforms of Gα_t or transducin (G$\alpha_{t\text{-}r}$ and G$\alpha_{t\text{-}c}$) are expressed exclusively in retinal rods and

cones, respectively, and link the photon receptor rhodopsin to the activation of a cyclic GMP phosphodiesterase. $G\alpha_{gust}$ is a taste receptor cell-specific G protein closely related to the transducins that mediates both bitter and sweet signal transduction (Wong et al., 1996).

$G\alpha_i$ and $G\alpha_o$ are involved in the regulation of signalling systems that can be disrupted by pertussis toxin (PTX), as they all serve as substrates for this toxin. PTX modifies covalently the α subunits by ADP-ribosylation of a cysteine residue four amino acids from the C-terminus (Birnbaumer et al., 1990). In contrast to the modification produced by CTX, this alteration results in the uncoupling of the modified G protein from its activating receptor(s) and therefore in the disruption of signal transduction. PTX-sensitivity is a property which has greatly helped the elucidation of the signals regulated by $G\alpha_i$ and $G\alpha_o$. The block of many cellular responses by PTX implies therefore an involvement of $G_{i/o}$ family members in a variety of signalling pathways, from the inhibition of adenylyl cyclase to the activation or inhibition of several ion channels. The $G\alpha_i$ and $G\alpha_o$ subunits interact with a large group of receptors (such as α2 adrenergic, M2 muscarinic, D2 dopaminergic, somatostatin, thrombin and LPA receptors) and regulate many cellular responses. It is well established that the three α_i proteins inhibit adenylyl cyclase (Birnbaumer, 1990). Several results have demonstrated that $G\alpha_{i2}$ is also involved in mitogenic signals. Mutationally activated $G\alpha_{i2}$ has been shown to deregulate cell growth in various systems

and to induce transformation in a specific cell model, Rat-1 fibroblasts (Gupta et al., 1992). In addition, mutations that activate $G\alpha_{i2}$ have been detected in tumors of the ovary and of the adrenal cortex (Lyons et al., 1990) and in nonfunctioning pituitary adenomas (Williamson et al., 1994). It has been proposed that these mutations convert the $G\alpha_{i2}$ gene into an oncogene, termed gip2 (Lyons et al., 1990). The intracellular events involved in the mitogenic action of $G\alpha_{i2}$ are not completely understood. In Rat-1 fibroblasts $G\alpha_{i2}$ seems able to activate mitogen-activated protein kinase (MAPK) (Pace et al., 1991; Gupta et al., 1992). Recent results have shown that this subunit might activate the MAPK pathway by activation of Rap1GAPII and reduction of intracellular GTP-bound Rap1 (Mochizuchi et al., 1999).

Additional information on the function of $G\alpha_{i2}$ comes from the analysis of transgenic animal models. Disruption of the murine gene encoding the α_{i2} subunit causes mice to develop an inflammatory bowel disease resembling human ulcerative colitis and also adenocarcinomas of the colon (Rudolph et al., 1995). Furthermore, using cells from transgenic mice harbouring inducible expression of RNA antisense to the gene encoding $G\alpha_{i2}$, Moxham and Malbon (1996) showed that $G\alpha_{i2}$ deficiency in adipose tissue and liver produces hyperinsulinemia, resistance to insulin and impaired glucose tolerance. These effects are associated with increased protein-tyrosine phosphatase activity and attenuated insulin-stimulated tyrosine phosphorylation of insulin-receptor substrate 1(IRS-1). The authors

suggested therefore that $G\alpha_{i2}$ deficiency might be a model for insulin resistance characteristic of non-insulin-dependent diabetes mellitus, implicating $G\alpha_{i2}$ as a positive regulator of insulin action.

G α_o subunit

Strathmann et al. (1990) isolated cDNA clones encoding two forms of the G α_o subunit from a mouse brain library. These appear to be the products of alternative splicing. Tsukamoto et al. (1991) likewise concluded that two different $G\alpha_o$ mRNAs may be generated by alternative splicing of a single gene. The two forms have been named α_{o1} and α_{o2} (Price et al., 1990; Strathmann et al., 1990; Hsu et al., 1990 and Murtagh et al., 1991). These two subunits differ for 26 residues that are present only in α_{o2}. Some tissues contain multiple $G\alpha_o$ mRNAs of various sizes that differ in the 3-prime untranslated regions (UTRs). Murtagh et al. (1991) concluded that the $G\alpha_o$ mRNAs with different 3-prime UTRs arise by alternative splicing of transcripts from a single gene. The UTRs were shown to exhibit a high degree of interspecies conservation and may play a role in regulation of $G\alpha_o$ expression during differentiation or in specific tissues. Very recently a third $G\alpha_o$ isoform, termed $G\alpha_{o3}$, has been purified from mammalian brain (Exner et al., 1999). It has been suggested that $G\alpha_{o3}$ originates from an enzymatic deamidation of $G\alpha_{o1}$ by an as yet-unidentified activity. The possible functional differences between the various $G\alpha_o$ subunits have not been

clearly established. However, it has been suggested that $G\alpha_{o1}$ and $G\alpha_{o2}$ might couple to different receptors (Kleuss et al., 1991). $G\alpha_o$ has unique and interesting properties. It is the most abundant G protein α subunit present in neurons, where it can constitute up to 2% of the total membrane protein (Huff et al., 1985; Giershik et al., 1986; Asano et al., 1989). In addition to neurons, $G\alpha_o$ appears to be expressed, albeit at much lower levels, in endocrine cells, heart, testis, skeletal muscle and uterus. Little or no α_o can be detected in liver, kidney and spleen (Price et al., 1989; Strathmann et al., 1990). Among cell lines, the protein is found in PC12, GH_3, and CHO cells but not in COS7 cells (Wu et al., 1992; Law et al., 1993). This pattern of expression predicts that α_o may play an important role in neuronal function. Indeed, analysis of transgenic mice lacking $G\alpha_o$ has revealed that these animals suffer tremors and seizures, are hyperalgesic and display a severe motor control impairment (Valenzuela et al., 1997; Jiang et al., 1998). Jiang et al. (1998) disrupted the G α_o gene in mice by homologous recombination, median survival was only 7 weeks. At the cellular level, inhibition of cardiac adenylyl cyclase by carbachol was unaffected, but opioid receptor-mediated inhibition of calcium channel currents was decreased by 30%. In 25% of the homozygous mutant cells examined, the calcium channel was activated at voltages lower than in their counterparts. Loss of $G\alpha_o$ was not accompanied by appearance of significant amounts of active free $\beta\gamma$ dimers. Homozygous

mutant mice were hyperalgesic and displayed a severe motor control impairment. Despite this problem, homozygous mutant mice were hyperactive and exhibited a turning behavior that had them running in circles for hours on end both in cages and in open-field tests. Except for one, all mutant mice turned counterclockwise. These results indicated that $G\alpha_o$ plays a major role in motor control, motor behavior, and pain perception and predict involvement of $G\alpha_o$ in calcium channel regulation.

To analyze the function of $G\alpha_o$ in the heart, Valenzuela et al. (1997) generated knockout mice lacking both forms of $G\alpha_o$ by homologous recombination and studied the muscarinic regulation of calcium channels in cardiac muscles in $G\alpha_o$ -/- mice and controls. There was no difference in the effect of isoproterenol on the L-type voltage-dependent calcium channel in ventricular myocytes of both groups, but the inhibitory effect of carbamylcholine was almost completely abolished in the $G\alpha_o$ -/- group. This demonstrated that, in the heart, $G\alpha_o$ is specifically required for transmission of signals from the muscarinic receptor to the L-type voltage-dependent calcium channel.

Despite these data, knowledge of effector systems that may be the targets of activated $G\alpha_o$ is still very limited. For a long time, $G\alpha_o$ was thought to be involved in the regulation of N-type voltage-dependent Ca^{2+} channels. However, it has been recently elucidated that inhibition of these channels by

G_o is mediated by the $\beta\gamma$ and not the α subunit of the heterotrimer (Ikeda, 1996; Herlitze et al., 1996; Qin et al., 1997). In contrast, it is well established that α_o can directly inhibit a specific adenylyl cyclase form, type I, which is mainly expressed in brain (Sunahara et al., 1996). There is also evidence that $G\alpha_o$ is able to regulate mitogenic signals and even to induce transformation: indeed, Kroll et al.(1992) demonstrated that expression of Q205L $G\alpha_o$, which lacks guanosine triphosphatase activity in NIH3T3 cells, results in transformation in a phospholipase C-independent manner. In CHO cells, it has been proposed that, the muscarinic M1 and PAF receptors, which couple to both G_q and G_o, activate a $G\alpha_o$-dependent pathway leading to MAPK stimulation (van Biesen et al., 1996). Recent studies (Antonelli et al., 2000) have shown in the same cell system that activated $G\alpha_o$ is unable to induce MAPK activation but strongly potentiates the stimulatory effects of the EGF receptor. This effect was not associated with changes in the state of tyrosine phosphorylation of the EGF receptor. The activated mutant $G\alpha_o$-Q205L also potentiated MAPK stimulation by activated Ras. In CHO cells, EGF receptors activate B-Raf, but not Raf-1 or A-Raf. It was found that expression of activated $G\alpha_o$ stimulated B-Raf activity independently of the activation of the EGF receptor or Ras. Inactivation of protein kinase C and inhibition of phosphatidylinositol-3 kinase abolished both B-Raf activation and EGF receptor-dependent MAPK stimulation by G α_o. Moreover, G α_o

failed to affect MAPK activation by fibroblast growth factor receptors, which stimulate Raf-1 and A-Raf but not B-Raf activity. These results suggest that $G\alpha_o$ can regulate the MAPK pathway by activating B-Raf through a mechanism that requires a concomitant signal from tyrosine kinase receptors or Ras to efficiently stimulate MAPK activity (Fig. 3). Further experiments have shown that receptor-mediated activation of $G\alpha_o$ caused a B-Raf response similar to that observed after expression of the mutant subunit. The finding that $G\alpha_o$ induces Ras-independent and PKC- and PI3 kinase-dependent activation of B-Raf and conditionally stimulates MAPK activity provides direct evidence for intracellular signals connecting this G protein subunit to the MAPK pathway. Another recent study has shown that G α_o can directly interact with Rap1GAP (Jordan et al. 1999). The authors propose that $G\alpha_o$ in its resting state sequesters Rap1GAP and, once activated, releases this regulatory protein, thereby controlling Rap1 activity. Finally, by parallel experiments, Ram et al. (2000) studied the roles of the MAP kinases and Stat3 in transformation of NIH3T3 cells by $G\alpha_o$-Q205L. Expression of $G\alpha_o$-Q205L in NIH3T3 cells activated Stat3 but not MAPK1 or 2. Coexpression of dominant-negative Stat3 (Signal transducer and activator of transcription 3) inhibited $G\alpha_o$-Q205L-induced transformation of NIH3T3 cells and activation of endogenous Stat3. Furthermore, $G\alpha_o$-Q205L expression increased activity of the c-Src, and the $G\alpha_o$-Q205L-induced activation of

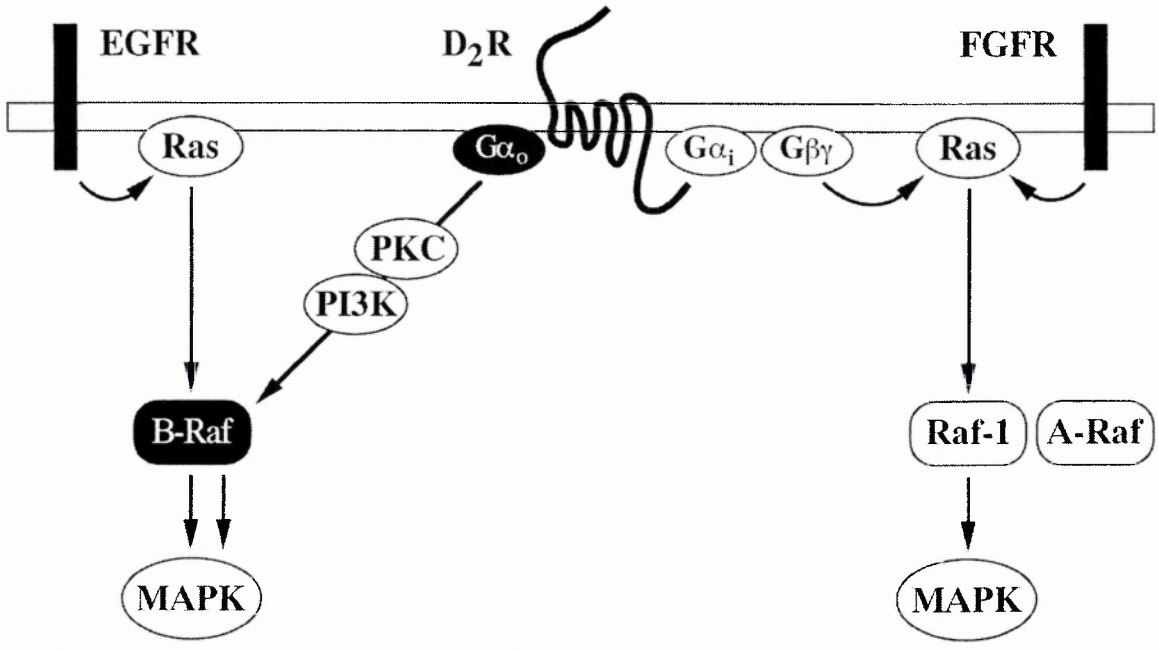


Fig.3 Model of signalling pathways regulating Raf and MAPK in CHO cells. The EGF receptor (EGFR) activates Ras and B-Raf, whereas the FGF receptor (FGFR) activates Ras, Raf1, and A-Raf. Activated $G\alpha_o$ potentiates MAPK activation by Ras and EGFR but not FGFR. The G protein subunit stimulates the kinase activity of B-Raf by a Ras-independent and PKC-and PI3K-dependent mechanism, which is insufficient to promote MAPK activation. It is proposed that this mechanism can efficiently stimulate the MAPK pathway in the presence of a B-Raf-activating signal from Ras or the EGFR. The dopaminergic D_2 receptor (D_2R), which couples to both G_i and G_o, appears to stimulate MAPK through Ras activation of Raf-1. This effect is presumably mediated by G protein $\beta\gamma$ complexes and, possibly, $G\alpha_i$ subunits. Upon stimulation of this receptor, the Ras-independent and PKC- and PI3K-dependent activation of B-raf induced by $G\alpha_o$ does not produce a MAPK response.

Stat3 was blocked by expression of CSK, which inactivates c-Src. Ram et al. (2000) concluded that Stat3 can function as a downstream effector for $G\alpha_o$-Q205L and mediate its biological effects. Regulation of B-Raf, Rap1GAP, the Stat3 and MAPK pathways might explain how $G\alpha_o$ can regulate cell growth. Other reported effects of $G\alpha_o$ include stimulation of polyphosphoinositide hydrolysis and PKC activity in *Xenopus* oocytes (Moriarty et al., 1990; Kroll et al., 1991), activation of a Src-like kinase pathway in neurons (Diversè-Pierluissi et al., 1997) and inhibition of cardiac L type Ca^{2+} currents (Jiang et al., 1998). How these effects come about has not been established. Cumulatively, these studies suggest that, in order to elicit its wide biological effects, $G\alpha_o$ may be able to engage into several distinct signalling pathways.

Finally, it is worth mentioning that there is evidence that $G\alpha_o$ might be activated not only by seven transmembrane domain receptors but also by other proteins: GAP43, an intracellular growth cone-associated protein involved in neurite outgrowth (Strittmatter et al., 1990); and the Alzheimer amyloid protein precursor protein responsible for familial forms of this disease (Nishimoto et al., 1993). Additionally, it has been proposed that $G\alpha_o$ is not only involved in signal transmission but also in the regulation of the secretory process. In C6 glioma cells, $G\alpha_{o1}$ has been detected on the membrane of small intracellular vesicles and appears to regulate secretion of the protease nexin-1 (Lagriffoul et al. 1996). In chromaffin cells, $G\alpha_o$

associated with secretory organelles has been proposed to regulate the ATP-dependent priming of exocytosis, by affecting the actin cytoskeleton. This effect might be related to the Rho-dependent activation of a PI4K localized on the membrane of secretory granules (Gasman et al., 1998). Furthermore, in PC12 cells the $G\alpha_{o2}$ subunit present on the membrane of secretory vesicles, seems to control catecholamine uptake (Ahnert-Hilger et al., 1999). These results suggest a function of $G\alpha_{o2}$ in regulation of neurotransmitter storage.

Light response of retinal ON bipolar cells seems to be regulated by $G\alpha_o$ subunit. Retinal ON bipolar neurons detect the glutamate released by rods and cones via metabotropic glutamate receptor 6 (mGluR6), whose cascade is unknown. By studies of the retina in $G\alpha_o$ -/- mice, Dhingra et al. (2000) found that the b-wave of the electroretinogram, both rod- and cone-driven components, was entirely missing. Further electroretinography performed on mice deficient for one splicing variant or the other have shown that the positive b-wave requires α_{o1} but not α_{o2} (2002).

Parallel work by Chen and Lambert was focused on the analysis of the $G_{i/o}$ proteins-mediated presynaptic inhibition at rat hippocampal synapses. By adenoviral expression of mutant PTX-insensitive subunits in cultured cells, they found that α_o rescued adenosine-induced presynaptic inhibition and the rapid onset of the response was mediated by a RGS protein. By two-yeast hybrid system, Traver et al. (2000) isolated a novel RGS protein, RGS14,

which is highly expressed in hippocampus; deletion analysis as well as in vitro binding experiment have shown that RGS14 is able to bind to Rap protein and, over other Gα subunits, preferentially to α_o . Very recently, Straiker et al. (2002) have confirmed previous results from Chen and Lambert by the same experimental approach.

In the heart, reactive oxygen species (ROS) have important functions in ischaemia/reperfusion-induced cardiac injury and in cytokine-stimulated hypertrophy. Extracellular signal-regulated kinase (ERK) is one of the ROS-responsive serine/threonine kinases. In vitro studies by Nishida et al. (2000) have found that treatment of rat neonatal myocytes with H_2O_2 activates $G\alpha_o$ and $G\alpha_i$ subunits ina receptor independent manner, leading to the increased activity of ERK. Further mass spectrometry studies have demonstrated that H_2O_2 effect consists of two steps: the first step is the modification of Cys^{287}, leading to subunit dissociation. The second step is the modification of Cys^{326}, resulting in the increased rate of GDP dissociation (Nishida et al., 2002).

3) **$G\alpha_q$**: this class includes $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15/16}$ which mediate phospholipase C (PLC) activation by membrane receptors such as the α1 adrenergic, the M1 muscarinic and the TRH receptor.

$G\alpha_q$ is a 42 kDa protein originally purified from liver plasma membranes (Taylor et al., 1990) and cloned from mouse brain (Strathmann and Simon,

1990). Recent studies of Bence et al. (1997) have revealed that, in addition to PLC, $G\alpha_q$ can directly stimulate the non-receptor tyrosine kinase Btk.

G_q and G_{11} are widely distributed in mammalian tissues, as demonstrated at the mRNA (Strathmann and Simon, 1990) and protein (Pang and Sternweis, 1990) levels. Studies on $G\alpha_q$ in transgenic mice have shown that this subunit is particularly important in platelet function. Disruption of the $G\alpha_q$ gene provokes unresponsiveness of the platelets to a variety of physiological activators, thus leading to increased bleeding times and protection from collagen and adrenaline-induced thromboembolism (Offermanns et al., 1997a). $G\alpha_{14}$ is most abundant in spleen, lung, kidney and testis, whereas $G\alpha_{15}$ and $G\alpha_{16}$ are confined to the hematopoietic cells (Wilkie et al., 1991; Amatruda et al., 1991).

4) $\mathbf{G\alpha_{12/13}}$: $G\alpha_{12}$ and $G\alpha_{13}$ are ubiquitously expressed and appear involved in several signalling pathways. They can elicit various responses: activation of both the Cdc42/Rac and the Jun kinase pathway; stimulation or inhibition of the MAPK pathway and of the Rho-dependent regulation of the cytoskeleton. The effectors and mechanisms of action of α subunits of these G proteins have not been clearly established. Jiang et al. (1998) have shown that $G\alpha_{12}$ binds directly to, and stimulates the activity of the tyrosine kinase Btk and of a Ras GAP, Gap1m. Other studies have revealed that the GTPase activities of

$G\alpha_{12}$ and $G\alpha_{13}$ are stimulated by the Rho guanine nucleotide-exchange factor p115 RhoGEF (Hart et al., 1998; Kozasa et al., 1998). Activated $G\alpha_{13}$ binds tightly to p115 RhoGEF and enhances its stimulation of nucleotide exchange on Rho. In contrast, activated $G\alpha_{12}$ inhibits this stimulation by $G\alpha_{13}$. Thus, p115 RhoGEF may act as an intermediary in the regulation of Rho proteins by G_{12} and G_{13}.

Disruption of the gene encoding $G\alpha_{13}$ in mice impairs the ability of endothelial cells to develop into an organized vascular system, with ensuing intra-uterine death (Offermanns et al., 1997b). In addition, $G\alpha_{13}$ (-/-) embryonic fibroblasts show greatly impaired migratory responses to thrombin. These results demonstrate that $G\alpha_{13}$ participates in the regulation of cell movement in response to specific ligands as well as during developmental angiogenesis.

G protein $\beta\gamma$ subunits

Five β subunits and more than ten γ subunits have been identified (Simon et al., 1991; Clapham et al., 1997). With the exception of the brain specific β_5 subunit, which is only 50% identical to the others, β subunits show a high degree of homology (79-90%) (Watson et al., 1994). β subunits migrate as 35-36 kDa proteins and are composed of seven repetitive segments of

approximately 40 amino acids that contain a characteristic tryptophan-aspartic acid pair (WD-40 repeats) (Neer, 1995; Hamm, 1998). By contrast, G protein γ subunits (5-10 kDa) are more heterogeneous. Functional differences between βγ complexes may thus be primarily due to the nature of the γ subunit. Most β and γ subunits appear to be able to form complexes; a few combinations, however, are never formed (Neer, 1995; Clapham and Neer, 1997). In addition, it is not clear whether within the cell the various α subunits associate randomly with βγ complexes, generating many different heterotrimers, or whether preferred combinations of Gα and Gβγ do take place. A great diversity in the number of heterotrimer combinations has been observed (Wilcox et al., 1995). Experiments involving synthesis inhibition of specific Gα, Gβ or G γ subunit suggest that defined αβγ combinations carry out specific functions (Kleuss et al., 1992, 1993). Some α subunits are myristoylated at the N-terminal glycine (α_o, α_i, α_z) and this modification is necessary for both membrane attachment and facilitated binding of βγ (Neer, 1995). The γ subunits differ from each other in the prenyl group bound to their C-terminal cysteine (Neer, 1995); prenylation and/or further carboxyl-terminal processing of γ are not necessary for βγ formation, but are indispensable for high affinity interactions of βγ with either G protein α subunits or adenylylcyclases (I-iguez-Lluhi et al., 1992).

The role of the βγ complex was long thought to be limited to the regulation

of GDP dissociation from Gα and the facilitation of Gα activation by receptors. In the case of $G_{i/o}$ proteins, this pathway is disrupted by PTX as $G_{i/o}$ α subunits are ADP-rybosilated by this toxin which prevent them from coupling to the receptor. More recent evidence has clearly established that free Gβγ can regulate specific effectors. In fact, the Gβγ complex stimulates phospholipase C, stimulates or inhibits enzymes of the adenylyl cyclase family, activates PI3Kγ, activates inwardly rectifying K^+ channels and inhibits N-type Ca^{2+} channels (Neer, 1995; Clapham and Neer, 1997; Hamm, 1998). Yan and Gautam (1996) have shown that the β and not the γ subunit interacts with adenylyl cyclase type II and the inwardly rectifying K^+ channel, GIRK1. The region involved in the interaction is a specific N-terminal fragment of 100 residues present in both target proteins.

A primary role of the βγ complex in the stimulation of the MAPK cascade has also been proposed by several studies (Hamm, 1998). Gβγ can activate PI3Kγ which is able to increase the activity of a Src kinase leading to tyrosine phosphorylation of Shc (src homolog and collagen), formation of a Shc-Grb2 (growth factor receptor binding protein) complex and Ras-dependent activation of the MAPK cascade (van Biesen et al., 1996b; Gutkind, 1998).

Other potential Gβγ effectors include the non receptor tyrosine kinases, Btk and Tsk, and the membrane-bound enzyme phospholipase A2 (Clapham and Neer, 1997; Hamm, 1998). It has also been reported that βγ subunits

negatively regulate dynamin I GTPase activity (Lin and Gilman, 1996) and interact with Raf1 (Pumiglia et al., 1995). It should be finally mentioned that another role of the βγ complex in cellular signalling is the membrane targeting of the β-adrenergic receptor kinase, βARK, involved in desensitization of the receptor response (Touhara et al., 1994). Several studies have addressed the question of functional differences between different βγ combinations with regard to effector regulation. With the exception of $\beta_1\gamma_1$, which is only found in the retina, no profound differences between βγ complexes have been detected (Neer, 1995; Hamm, 1998). Some recent data support, however, a specialized role for the β_5 subunit in cell signalling. It has been shown that the $\beta_5\gamma_2$ dimer differs from dimers containing the β_1 subunit in the specificity of effectors (Lindorfer et al., 1998). Further studies have revealed that, after expression in Sf9 cells, $G\beta_5$, but not $G\beta_{1\text{-}4}$, forms cytosolic complexes with RGS6 and RGS7 which, unlike conventional Gβγ complexes, do not form heterotrimeric complexes with either $G\alpha_o$-GDP or $G\alpha_q$-GDP, but are able to inhibit $\beta_1\gamma_2$-mediated activation of PLC β2 (Posner et al., 1999). The precise role of RGS/β_5 complexes in signal transduction remains, however, to be established.

PHOSPHOINOSITIDE HYDROLYSIS

Upon binding to their cell surface receptors, many extracellular signalling

molecules including neurotransmitters, hormones, growth factors, and immunoglobulins elicit intracellular responses by activating PLC. Activated PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) with generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Diacylglycerol is a direct activator of protein kinase C (PKC) (Nishizuka et al., 1992), whereas IP_3 induces release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm (Berridge, 1993). This bifurcating pathway lies at the root of a transmembrane signal transduction mechanism that regulates a large array of cellular processes, including metabolism, secretion, contraction, neuronal activity, and proliferation. The second messenger IP_3 has a key role in controlling both the mobilization of internal stores and the entry of external calcium. Cells generate IP_3 through two major signalling pathways. In one pathway, receptors are coupled through G proteins to stimulate PLC β, whereas the other pathway depends upon tyrosine kinase-linked receptors which are specifically coupled to PLC γ.

PHOSPHOLIPASE C

Direct protein isolation and molecular cloning studies have revealed the existence of multiple PLC isozymes in mammalian tissues (Rhee et al., 1989; Rhee et al., 1992). As predicted from the cDNAs, the PLC isozymes vary in size, with molecular masses ranging from 85 to 260 KDa. Despite the low

overall homology among the amino-acid sequences, significant similarity is apparent in two regions (one of about 170 residues and the other of about 260 residues) that are designated as the X and the Y domains. These domains appear essential for the catalytic activity of the enzymes (Meldrum et al., 1991; Lee and Rhee, 1995). Eighty amino-acids are either invariant or conservatively changed in each of the X and Y regions of many PLC sequences, whereas only 8 amino-acids are conserved outside these two regions. Because all of the latter 8 conserved amino acids are located at the amino-terminal side of the X region, the line of demarcation of the start of the X region is not clear-cut. All PLCs contain an N-terminal region of about 300 amino acids that precedes the X region and contains a pleckstrin homology (PH) domain.

On the basis of the relative locations of the X and Y domains in the primary structure, PLC isozymes are classified into four types: β, γ, δ and ε (Fig.3). Interestingly, the C-terminal sequence following the Y domain is about 450 aminoacid long in PLC-β and is almost non-existent in PLC- δ. The PLC originally defined as α in sheep seminal vesicles is probably a proteolytic product of a δ isozyme (Exton, 1996), and the cDNA originally reported for PLC α encodes a protein-disulfide isomerase (Exton, 1996).

All four types of PLC catalyze the hydrolysis of the three common inositol containing phospholipids: phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), and PIP_2, though PIP and PIP_2 are the preferred

substrates. PIP and PIP_2 are produced by phosphorylation of phosphatidylinositol. In most cell types, PIP_2 constitutes less than 10% of the total inositol lipids and less than 1% of the total phospholipids. Although all three inositol phospholipids may be broken down in the signalling response, the breakdown of PIP_2 is most critical, even though it is the least abundant (Ryu et al., 1987). The catalytic activities of all three types of PLC are dependent on Ca^{2+}. The δ-type isozymes are more sensitive to this cation. Structural and mutational studies have identified residues within the catalytic domain that likely contribute to substrate recognition, Ca^{2+} binding, and catalysis (Ellis et al., 1998). The crystal structures of complexes of PLC δ1 with Ca^{2+} and $Ins(1,4,5)P_3$ reveal the complex interactions of Ca^{2+} with several negatively charged residues (Glu^{3441}, Asp^{343}, and Glu^{390})and Asn^{312} in the active site as well as with the 2-hydroxyl group of $Ins(1,4,5)P_3$ (Essen et al., 1996; Ellis et al., 1998).The importance of all four these amino acid residues in coordination of Ca^{2+} was demonstrated by analysis of the PLC activity of the respective point mutants at various concentrations of Ca^{2+} (Ellis et al., 1998).

The C2 domains of PLC isozymes comprise ~120 residues, and such domains are present in many proteins that interact with lipid membranes. The C2 domain of PLC δ1 has been estimated to contain three to four Ca^{2+} binding sites. Calcium ions bound to the C2 domain of were recently shown to enhance enzyme activity (Lomasney et al., 2001). In contrast, the C2

domains of PLC β isozymes exhibit no apparent affinity for membrane bilayers in the presence of Ca^{2+}, but they do interact with activated α subunits of G_q proteins (Wang et al., 1999).

The role of the EF-hand domains of PLC isozymes is not clear. Crystallographic data suggest that the EF-hand domain of does not bind Ca^{2+} but rather serves as a flexible link between the PH domain and the rest of the enzyme, thus allowing the C2 and catalytic domains to interact with the membrane after binding of the PH domain to PtdIns(4,5)P_2 (Ellis et al., 1998; Essen et al., 1997). However, more recent evidence indicates that the EF-hand domain of PLC δ1 does bind Ca^{2+} and that the bound Ca^{2+} is necessary for the efficient interaction of the PH domain with PtdIns(4,5)P_2 (Yamamoto et al., 1999).

The different types of PLC differ in their subcellular localization. When tissues are homogenized in low ionic strength buffers, PLC β isozymes are recovered predominantly in the particulate fraction whereas PLC γ and PLC δ are detected mainly in the cytosolic fraction (Exton, 1996). Association of PLC γ1 with the cytoskeleton has also been described (Mc Bride et al., 1991; Yang et al., 1994). Several studies have shown localization of PLCs in nuclei (Exton, 1996). In particular, PLC β1 has been reported to be localized exclusively in the nucleus of Swiss 3T3 cells (Martelli et al., 1992). The physiological role and activation mechanism of nuclear PLCs are unclear.

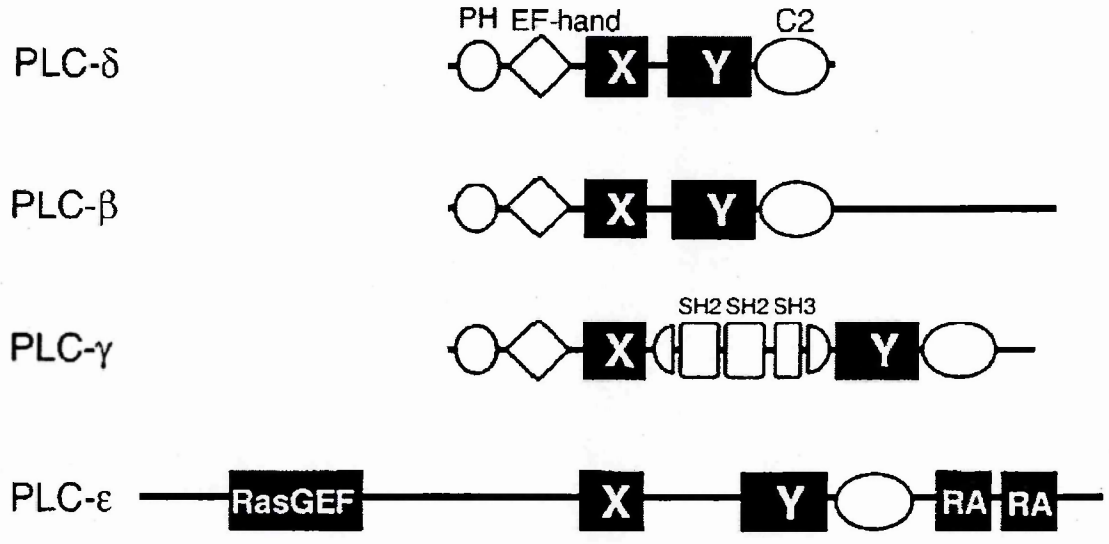


Fig.4. Phospholipase C family. Domain organization of the four types of PLC isozymes. The X and Y catalytic domains as well as the PH, EF-hand, C2, SH2, SH3, RasGEF, and RA domains are indicated (Rhee SG, 2001).

The various PLC families differ in their modes of activation in response to extracellular stimuli. PLC β enzymes are regulated by G proteins and PLC γ by tyrosine kinases. At the moment the mechanism of regulation by receptors at the cell surface of PLC δ is still unclear or even if it is at all; it is possible that PLC δ enzymes are regulated only by calcium ions (Irvine, 1996). Although the various members of each family can be distinguished by their structural features and their expression patterns, it is not clear what roles the isoenzymes of a given PLC family play in vivo, or to what extent they determine the specificity of individual signalling pathways.

In this thesis, PLC-γs, PLC-δs and PLC-εs will be described only briefly, while the PLC β isozymes involved in G protein signalling pathway will be discussed in detail.

PLC γ

The PLC-γ enzymes are activated by polypeptide growth factors, that bind to receptors with an intrinsic tyrosine kinase activity, such as PDGF, EGF, FGF and NGF (Rhee and Choi, 1992; Pawson and Schlessinger, 1993). Other receptors that lead to the activation of PLC γ are not protein kinases themselves but activate intracellular non-receptor tyrosine kinases (Lee and Rhee, 1995). Such receptors include the T cell antigen receptor, the membrane associated IgM, the high affinity IgE receptor, the IgG receptors and several receptors for cytokines.

Activation of PLC γ by growth factors has been studied in many cellular systems (Pawson and Schlessinger, 1993). Binding of the growth factors to their receptors results in receptor homodimerization and activation of the intrinsic tyrosine kinase activity. The phosphotyrosine residues of the autophosphorylated receptors provide high affinity binding sites for several proteins containing Src homology SH2 domains. These include PLC-γ. Whilst PLC- β and PLC- δ contain short sequences of 50-70 amino acids separating the X and Y regions, PLC- γ has a long sequence of about 400 amino acids which contains the so called Src homology SH2 and SH3 domains (Koch et al., 1991) (see Fig.3). Bound PLC γ is activated by the tyrosine kinase receptor through tyrosine phosphorylation (Rhee and Choi, 1992; Exton, 1996).

PLC γ is one of many signalling proteins known to be activated upon stimulation by growth factors. Several studies have shown that PLC γ activation and the consequent increase in second messenger production contributes to cell proliferation in response to growth factors (Rhee and Choi, 1992; Exton, 1996). In addition, several lines of experimental evidence suggest that PLC γ may regulate actin polymerization by reducing the concentration of PIP2 (Exton, 1996).

Two PLC isozymes participate in these pathways: PLC- γ1 and - γ2 (Lee and Rhee, 1995). PLC- γ1 has been studied more extensively than PLC- γ2, which is mainly expressed in haemopoietic cells. Both the enzymes appear to

be activated by non receptor PTKs, even if the ratio of PLC-γ2 to PLC-γ1 concentrations and the relative extents of phosphorylation differ significantly between the respective haemopoietic cell types (Rhee and Choi, 1992).

Up-regulated signaling from the epidermal growth factor receptor (EGFR) has been correlated with tumor invasion and metastasis in numerous human neoplasias. Turner et al. (1997) have demonstrated that increased levels of EGFR promote invasiveness of human prostate carcinoma DU-145 cells. To analyse the increased cell-motility signaled via PLC γ activation, highly invasive DU-145 cells engineered to overexpress the EGFR were stably transfected with **PLC z**, a dominant-negative fragment of PLC γ which consists of SH2/SH3 domains and an inhibitor (I) domain (Homma et al., 1992). Tumors from the cells expressing PLC z fragment were significantly less invasive than control cells. Similar results have been obtained recently in bladder carcinoma cell lines (Kassis et al., 2002). These data suggest that PLC γ could represent a potential therapeutic target to limit tumor progression promoted by up-regulated signaling from the EGFR and related receptors with intrinsic tyrosine kinase activity.

PLC δ

In contrast to the PLC γ isozymes, the receptor-linked activation mechanism(s) of PLC δ has not been fully clarified. So far the family

includes four mammalian isozymes: δ1, δ2, δ3 and δ4. Molecules similar to members of the PLC δ family are the only PLC types that have been isolated from simple organisms such as yeast and slime mould (Exton, 1996). PLC δ is insensitive to the activation of G α_q, G βγ or tyrosine phosphorylation (Exton, 1996). As described above, the sensitivity of PLC δ isozymes to Ca^{2+} is greater than of the other isozymes. Calcium promotes both the association of the PH domain with PtdIns(4,5)P2 as the result of its binding to the EF-hand domain (Yamamoto et al., 1999) as well as the formation of a C2 domain-phosphatidylserine-Ca2+ ternary complex as the result of its binding to the C2 domain (Lomasney et al., 1999). Banno et al.(1994) revealed that thrombin stimulation of CHO cells overexpressing PLC- δ1 is able to induce activation of the enzyme via both G proteins and Ca^{2+}. The activity of both PLC δ1 and PLC δ4 is clearly stimulated by Ca^{2+} (Lee and Rhee, 1996). Recent studies have demonstrated by using a peptide approach and site-directed mutagenesis, that multiple regions of tissue type transglutaminase (TGII) interact with the α1B-AR and that the α1B-AR (alpha 1B adrenoreceptor) stimulates PLC δ via TGII (Feng et al., 1999). This novel potential regulator of PLC δ isozymes was termed high molecular weight G protein or G_h (75-80 kDa). This protein, which also possesses tissue transglutaminase activity, forms a complex with PLC δ1 in cells stimulated through α1-adrenergic, oxytocin receptor or interacts with thromboxane A2

receptor, increasing PLC activity. Recently it has been shown that the free or GDP-bound form of G_h are associated to PLC δ1 (Murthy et al., 1999), which suggests that the activity of PLC δ1 might be inhibited by G_h in unstimulated cells and that such inhibition might be attenuated by the binding of G_h in response to receptor occupancy.

PLC ε

PLC ε protein is the homolog of PLC 210, which was initially identified from a *Caenorhabditis elegans* cDNA library and contains several domains not apparent in other known PLC isoforms. The recently identified human PLC ε has been detected in two alternative splicing forms with molecular sizes of 230 and 260 kDa. The mRNAs encoding these enzymes are present in a wide variety of human tissues, most abundantly in the heart. Both the variants differ from the other three types of isozymes in that they do not contain a PH and EF-hand domains but possess an NH_2-terminal Ras guanine nucleotide exchange factor (RasGEF)-like domain and at least one and pheraps two COOH-terminal Ras binding (RA) domains. There are many evidences which support a possible role for PLC ε as GDP-GTP exchange factor for, as well as an effector of, Ras (Gao et al., 2001). PLC ε also appear to be activated by $G\alpha_{12}$ (Lopez et al., 2001).

PLC β

The PLC β family includes four subtypes: β1, β2, β3 and β4. Structural studies have revealed that the long C-terminal domain, that characterizes all

these enzymes, mediates their membrane localization (Kim et al., 1996) as well as their interaction with G protein α_q subunits (Park et al., 1993; Wu et al., 1993). Amino acid analysis of PLC β isozymes predicts that most of the C-terminal domain is helical, and three regions in this domain contain a high proportion of basic residues that are highly conserved (Lee et al., 1993). Projection of the sequences of these three regions as helical wheels reveals clustering of the basic residues. The role of the C-terminus and the clustered basic residues in PLC β was investigated by substitution of clusters of basic residues in different regions (Kim et al., 1996): none of the basic clusters appeared critical for particulate association, but all contribute to the association, presumably by interacting with acidic residues in the particulate fraction. Other studies have shown that, in addition to the C-terminus, the PH domain, known to bind PIP_2, is able to mediate membrane localization of PLC β isozymes (Kim et al., 1996).

PLC β 1

Phospholipase C β1 exists as two polypeptides of 150 and 140 kDa and is encoded in rat brain by two distinct transcripts of 5.4 and 7.2 kb (Suh et al., 1988). A cDNA corresponding to the entire 5.4-kb transcript revealed an open reading frame that encodes a 1216-amino acid polypeptide (Suh et al., 1988). Subsequently, Bahk et al. (1994) isolated the cDNA corresponding to the entire 7.2-kb transcript from a rat brain cDNA library. The 7.2-kb transcript differs from the previously reported 5.4-kb transcript by possessing

both an additional 118 nucleotides located near the end of the coding sequence and a 1738-nucleotide extension of the 3'-flanking region. The presence of the 118-nucleotide insert in the 7.2-kb sequence gives rise to an open reading frame that codes for a 1173-aminoacid polypeptide (PLC β1b), the C-terminal sequence of which differs from that of the 1216-aminoacid polypeptide derived from the 5.4-kb transcript (PLC β1a). Analysis of PLC β1 genomic DNA revealed that PLC β1a and PLC β1b are derived from a single gene by alternative RNA splicing (Bahk et al., 1994).

Studies of expression with antibodies able to recognize the 150-kDa enzyme as well as the 140-kDa variant showed that both proteins are expressed in much higher concentrations in the brain than in other tissues (Jhon et al., 1993). Analysis of PLC β1-/- mice has revealed that this enzyme is particularly important in signal transduction by muscarinic acetylcholine receptors in the cerebral cortex and hippocampus. As a consequence, PLC β1-/- mice develop epilepsy (Kim et al., 1997).

PLC β2

The PLC β2 cDNA was cloned and sequenced from HL-60 cells using PLC β1 cDNA as a probe under reduced stringency hybridization (Park et al., 1992). The cDNA encodes a putative polypeptide of 1181 amino acids with a calculated molecular mass of 133 kDa. Its pattern of expression has not been extensively characterized, but appears to be very limited (Jhon et al., 1993; Ali et al., 1997).

As will be discussed below, PLC β2 can be efficiently activated by G βγ subunits (Katz et al., 1992; Wu et al., 1993). Wu et al. (1993) have found that the N-terminal half of the molecule contains the sequences required for activation by G βγ. The G βγ–binding region of PLC β2 has been more recently narrowed to 62 amino acids (residues Leu-580 to Val-641) (Kuang et al., 1996) (see Fig.3).

PLC β3

PLC β3 was cloned and sequenced from rat thyroid FRTL5 cells (Jhon et al., 1993). The apparent size of the enzyme is 132-kDa. A distinctive feature of PLC β3 is that the X and Y domains are separated by 121 residues compared with 70 and 76 residues in PLC β1 and PLC β2.

PLC β3 appears to be the most widely expressed member of the PLC β family, particularly abundant in brain, parotid gland, liver and uterus (Jhon et al., 1993). The precise functional role of PLC β3 remains to be defined. Weber et al. (1994) have found that the PLC β3 gene shows loss of expression in tumors from endocrine pancreas, the adrenal cortex, and the thyroid medulla, but the implications of this finding are not clear (Weber et al., 1994).

PLC β4

PLC β4 was identified by several groups using different approaches. Min et al. (1993a,b) purified a novel PLC from bovine cerebellum and identified this enzyme as PLC β4 by comparing the partial aminoacid sequences with the

sequences of other PLC isozymes and by analyzing immunological cross-reactivity. Kim et al. (1993) cloned and sequenced the cDNA corresponding to PLC β4 from a rat brain cDNA library. This encodes a putative polypeptide of 1176 amino acids, with a calculated molecular mass of 130 kDa. In parallel, Lee et al. (1993) purified from a retinal particulate fraction a PLC preparation that contained a major protein band with an apparent molecular mass of 130 kDa. Analysis of tryptic peptides derived from the 130-kDa protein revealed a sequence corresponding to the enzyme PLC β4. A search of a rat brain cDNA library by PCR with oligonucleotide primers based on common PLC aminoacid sequences resulted in the cloning of the full-length cDNA. Bovine PLC β4 cDNAs were also isolated by Ferreira et al. (1993) using conserved regions of the cDNA encoding the Drosophila PLC norpA (no receptor potential). Indeed, PLC β4 is highly homologous (50%; Kim et al., 1993) to the norpA protein (Pak et al., 1970). GTPase sequence motifs present in proteins of the GTPase superfamily are found conserved in PLC β4 as well as norpA but not in other phospholipases C (Ferreira et al., 1993). These motifs G1-G5 have been shown to be important for GTP binding and GTPase activity (Kaziro et al., 1991). Motifs G1-G4 are strictly conserved, while G5, which is poorly conserved or even absent in other GTPases as well, is partially conserved. Motifs G1-G3 are found in box X, while G4 and G5 are in box Y and extended box Y, respectively; thus all

five motifs are found in all four variants of the protein. Actually, the functional role of these conservative regions has not yet been clarified.

The expression pattern of PLC β4 has not been extensively characterized. The enzyme has been detected in brain, retina and pituitary gland (Alvarez et al., 1995). In the brain, PLC β4 mRNA is highest in the cerebellum and is almost negligible in the cerebral cortex and hippocampus (Tanaka and Kondo, 1994).

Four alternative bovine forms of PLC β4 expressed preferentially in the retina, have been identified. These variants are characterized by different N-terminal regions and by the presence or absence of 36 nucleotides within the region between boxes X and Y (Ferreira et al., 1993). Interestingly, a cDNA sequence predicting a human PLC β4 polypeptide of 1022 amino-acid residues (117 kDa) has been isolated from a human retina library by Alvarez et al. (1995). This PLC β4 variant lacks the 165-aminoacid N-terminal domain present in the rat brain enzyme, but has a distinctive exon 1 unique for human and bovine retina isoforms (Fig.5).

In addition to these variants, a novel form of rat brain PLC β4 has been recently isolated by Kim et al. (1998). This variant, named PLC β4b, has a shorter C-terminal tail compared with the previously identified enzyme (PLC β4a) (Kim et al., 1993; Ferreira et al., 1993; Lee et al., 1993). In PLC β4b, the C-terminal 162 amino acids of PLC β4a are replaced by 10 distinct amino acids. The comparison of the cDNA sequences of PLC β4a and PLC

β4b predicts that they may be generated by alternative processing of the mRNA.

In contrast to PLC β4a, which is a membrane-associated enzyme activated by G α_q, PLC β4b is cytosolic and insensitive to G α_q stimulation. Therefore it seems that the carboxyl-terminal 162 amino acids in PLC β4a are necessary for the association with the particulate fraction and activation by G α_q.

As Banno et al.(1994) had reported that a carboxyl-terminal truncated form of PLC β3 generated by calpain cleavage can be activated to a greater extent by brain G protein βγ subunits than the intact PLC β3, it was suggested that the carboxyl-terminal region of PLC β3 may inhibit its activation by G protein βγ subunits. Although it has been known that PLC β4a cannot be activated by G protein βγ subunits in vivo and in vitro, Kim et al. raised the question of whether G protein regulation of the 116-KDa PLC β4b might occur by βγ subunits. However they found that PLC β4b, having a short carboxyl-terminal tail, is nevertheless unresponsive to regulation by G protein βγ subunits. Therefore these results suggested that PLC β4 does not have the sequence motif necessary to be activated by G protein βγ subunits.

Expression of PLC β4 in the retina and its clear homology to norpA sequence implies that PLC β4 may play a role in the mammalian visual process. A mouse line that lacked PLC β4 was generated by Jiang et al. (1996) and the physiological significance of PLC β4 in murine visual function was

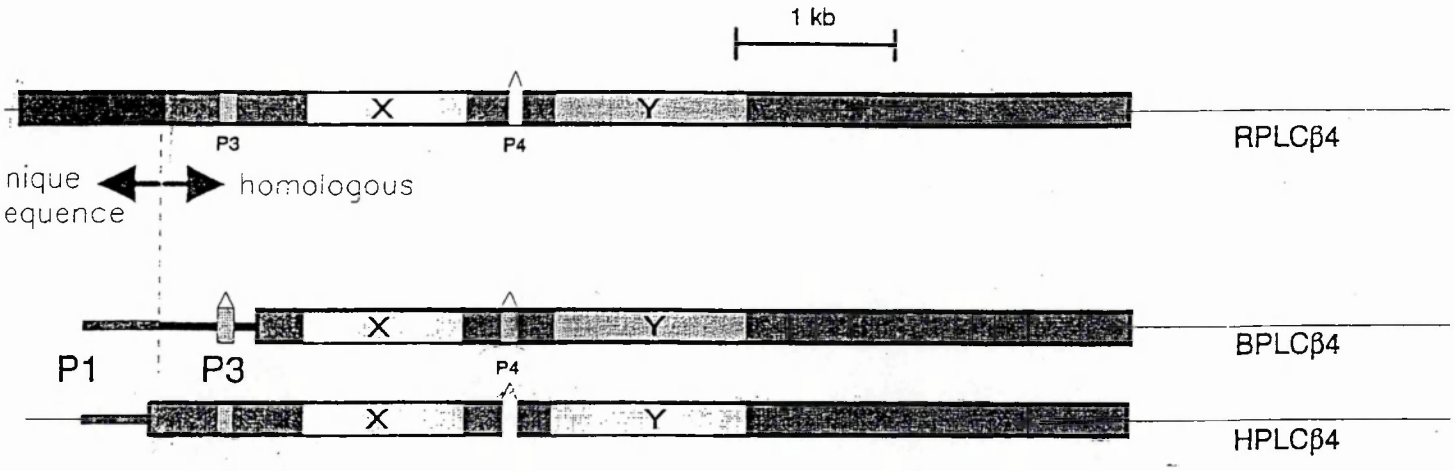


Fig.5 Domain structure of PLC β4 isoforms.

The rat brain (RPLCβ4; GenBank™/EMBL Data Bank accession number L15556), bovine retina (BPLCβ4; GenBank™/EMBL Data Bank accession numbers L13935, L13936, L13937, L13938), and human retina (HPLCβ4; GenBank™/EMBL Data Bank accession number L41349) isoforms are schematically represented by boxes. The isoforms are aligned according to the relative positions of alternatively spliced exons P3 and P4. Conserved X and Y boxes are lightly shaded. To the right of the dotted line, sequences are homologous in all three isoforms. To the left of the dotted line, sequences in the retina isoforms (p1 domain) are unique and specific. The P3 domain is contained in rat brain, human retina, and two of four bovine retina variants. The P4 domain has been identified only in bovine retina.

investigated. Behavioral tests using a shuttle box demonstrated that the mice lacking PLC β4 were impaired in their visual processing abilities, whereas they showed no deficit in their auditory abilities. In addition, the PLC β4-null mice showed 4-fold reduction in the maximal amplitude of the rod a- and b-wave components of their electroretinograms relative to their littermate controls. However, recording from single rod photoreceptors did not reveal any significant differences between the PLC β4-null and wild-type littermates, nor were there any apparent differences in retinas examined with light microscopy. These results suggest that the effect of PLC β4 deficiency on the rod signalling pathway occurs at some stage after the initial phototransduction cascade and may require cell-cell interactions between rods and other retinal cells. More recently, Kim et al. (1997) have reported that PLC β4-/- mice are ataxic. They were hypokinetic and showed a waddling gait, with the rear body swinging left-right. In the brain, expression of PLC β4 is highest in the cerebellum and is almost negligible in the cerebral cortex and hippocampus (Tanaka and Kondo, 1994). Histological analysis of PLC β4-/- mice revealed that the development of the cerebellum was retarded, but apparently normal except for a slight reduction in size. Dendrogenesis of Purkinje cells examined by anti-calbindin staining and the number of synapses on dendritic spines, as examined by transmission electron microscopy, were also normal in the cerebellum of PLC β4-/- mice, suggesting that the motor defect in these mice was not due to any grossly

abnormal development of the cerebellum. To determine the molecular basis of the phenotypic defects of the mutant mice, the authors selected three agonists on the basis of the known expression patterns of corresponding PLC-coupled receptors: carbachol (CCh), a non-selective agonist on muscarinic acetylcholine receptors (mAChR), 1-amino-1,3-cyclopentanedicarboxylicacid (ACPD), a non-selective agonist on metabotropic glutamate receptors (mGluR) and 1-(3-chlorophenyl)piperazine (mCPP), a non-selective agonist on serotonin (5-HT) type-2 receptors. In the cerebellum of PLC β4-/- mice there is less phosphoinositide hydrolysis induced by CCh or ACPD whereas PLC β1-/- mice show a preferential attenuation of CCh-induced phosphoinositide hydrolysis in the hippocampus. Thus, in cerebellum, PLC β4 appears to be the transducer of signals mediated by mGluR, whereas PLC β1 and PLC β4 both transduce mAChR-mediated signals. As PLC β4 is enriched in Purkinje cells, the ataxia phenotype of PLC β4-/- mice may, at least in part, be the result of an impaired mGluR signal-transduction pathway in Purkinje cells. This is supported by the findings that mGluR1 is expressed predominantly in cerebellar Purkinje cells (Masu et al., 1991) and that mGluR1 knockout mice show ataxia (Aiba et al. 1994). Thus, PLC β4 appears to be essential for transducing mGluR1-mediated signals in cerebellum, which accounts for the defect seen in PLC β4-/- mice. Activation of the type-1 metabotropic glutamate receptor (mGluR1) signaling pathway in the cerebellum involves activation of phospholipase C (PLC) and protein

kinase C (PKC) for the induction of cerebellar long term depression (LTD). In PLC β4-/- mice, recent work has shown that expression of total PLC β, the mGluR1-mediated Ca^{2+} response, and LTD induction are greatly reduced in rostral cerebellum. Furthermore, during LTD induction, both PKCα and PKCβ1 have been found to be translocated to the plasma membrane. These results suggest that mGluR1-mediated activation of PLC β4 in rostral cerebellar Purkinjie cells induces LTD via PKCα and/or PKCβ1 (Hirono et al., 2001).

NorpA

The norpA gene encodes at least four transcripts: a 7.5kb transcript that is expressed in eye and 6.5-, 5.5-, and 5.0-kb transcripts that are expressed in adult body or early stages of development. Antiserum generated against the major gene product of norpA recognizes a 130-kDa protein that is abundant in eyes but severely reduced or absent in norpA mutants; it also recognizes a 130-kDa protein in adult legs, thorax, and male abdomen, but not female abdomen. Furthermore, immunohistochemical staining of tissue sections with the norpA antiserum shows that the norpA protein is abundant in the retina and ocelli and is present to a lesser extent in the brain and thoracic nervous system (Zhu, 1993). This tissue distribution of norpA transcripts is identical to the distribution of transcripts from a *Drosophila* G_o alpha-subunit gene (Shortridge, 1991).

The norpA gene of *Drosophila melanogaster* encodes a phosphatidylinositol-specific phospholipase C that is essential for phototransduction, as its mutants have long been known to be deficient in light-induced electrical responses in the compound eye (Pak et al., 1970) and ocellus (Hotta and Benzer, 1970) with strong mutations rendering the fly blind. *Drosophila* photoreceptor neurons show a high degree of architectural organization, with most of the molecules involved in phototransduction localized to the rhabdomeres. In rhabdomeres, photoconversion of rhodopsin to metarhodopsin activates a G_q protein α-subunit ($G_q\alpha$), which in turn activates PLC β, leading to depolarization via gating of two cation channels, the transient receptor potential (TRP) and TRPL light-activated channels. Following termination of the stimulus, calcium-dependent regulatory processes including activation of protein kinase C (eye PKC), mediate deactivation of the light response. NORPA protein has been shown to be an integral part of a signal transduction complex associated with the rhabdomere membrane. This signal transduction complex is organized by an adapter protein, INAD (inactivation no-after potential D), which binds and clusters protein kinase C, the TRP calcium channel, and NORPA (PLCβ) in the visual cascade. The carboxyl terminal tail of NORPA interacts with the sequence spanning the last three PDZ domains of INAD (Shieh et al., 1997). Null INAD mutants have a dramatically reorganized subcellular distribution of signalling molecules, and a total loss of transduction complexes (Tsunoda

et al., 1997). Also, mutants defective in a single PDZ domain of INAD produce signalling complexes that lack the target protein and display corresponding defects in their physiology. Thus, the interaction of NORPA with INAD is essential for a rapid and controlled visual response.

The vertebrate retinal PLC, PLC β4, shares a greater similarity with NORPA than with other PLC β isotypes (Kim et al., 1993; Lee et al., 1993). This suggests that regulation of PLC β4-mediated signal transduction may be similar to the NORPA-mediated mechanism in *Drosophila*. PLC β4 is not involved in the rhodopsin initiated visual response in mice. However, PLC β4 is expressed in photoreceptor, bipolar, horizontal, and ganglion cells of the bovine retina, in the cerebellum and in neurons connecting the retina to the cerebellum in the human and mouse brain (Kim et al., 1993; Ferreira et al., 1994; Alvarez et al., 1995). The findings of Jang et al., (1996) and of Kim et al. (1997) by generating null PLC β4 mice indicate that PLC β4-mediated signalling is essential for processing of visual information required for learning from visual cues and coordination of ongoing movement. By analogy with NORPA-mediated signalling pathway, it is likely that the carboxyl terminal tail of PLC β4a interacts with an INAD-like adapter protein for inclusion in a membrane-associated complex, as it contains a PDZ binding consensus motif. It is interesting to note that a human INAD-like protein (hINADL) has been identified which is expressed in the cerebellum

(Philipp et al., 1997). Further analysis will be necessary to determine if indeed PLC β4a mediates a signalling cascade similar to that of NORPA.

MECHANISMS OF REGULATION OF PLC β BY G PROTEIN SUBUNITS

It is well established that both PTX-insensitive and PTX-sensitive G proteins couple receptors to PLC β (Fig.4). The PTX-insensitive pathway that stimulates PLC β is mediated by the G_q family. This pathway is activated by various receptors, including bradykinin, histamine, angiotensin, M1 muscarinic and α1 adrenergic receptors (Gutowski et al., 1991; Berstein et al., 1992b; Wu et al., 1992b). Several studies have shown that the α subunit of G_q proteins can directly stimulate PLC β isozymes (Taylor et al., 1991). Another group of agonists such as acetylcholine (binding to M2 and M4 muscarinic receptors), catecholamines (binding to α2 adrenergic receptors), interleukin 8, thrombin, eicosanoids (prostaglandin PGE2), the leukotrienes LTC4 and LTD4 and thromboxane TXA2, all activate PLC β via pertussis toxin-sensitive $G_{i/o}$ proteins (Dell'acqua et al.1993; Wu et al., 1993a). This activation is mediated by G βγ rather than G α subunit (Camps et al., 1992; Katz et al., 1992).

All the PLC β enzymes, with the exception of PLC β4b (Kim et al., 1998), can be activated by the α subunits of the G_q class (Smrcka et al., 1991; Taylor et al., 1991; Wu et al., 1992a; Jhon et al., 1993), but there is evidence that they differ in their sensitivity to the various α subunits. For instance, the β1 and β3 isozymes are activated to a similar extent by α_q, α_{11} and α_{16}, though the β2 isozyme appears less sensitive (Smrcka and Sternweis, 1993; Kozasa et al., 1993; Hepler et al., 1993). PLC β4 can be activated by all the α subunits of the G_q family, though α_q and α_{11} are the most efficient (Jiang et al., 1994).

There has been some definition of the domains in PLC β that are involved in interaction with α_q (Fig.3). Wu et al (1993b) coexpressed various mutant forms of PLC β1 with α_q in COS7 cells and found that the region required for α_q activation was localized to residues 1030-1142 of the C-terminal portion of the molecule (Wu et al., 1993b). These results were supported by the observation that two peptides from the interaction region inhibit activation of the enzyme by α_q. The conclusion that sequences of the C-terminal region of PLC β1 are involved in its interaction with α_q is reinforced by the analysis of the ability of proteolyzed forms of the phospholipase to interact with the α subunit (Park et al., 1993). These studies localized the activation site between residues 880 and 1130. Additionally, Blank et al. (1993) found that a truncated form of PLC β3 that lacked most of the C-

terminus was completely unresponsive to $\alpha_{q/11}$, similarly to PLC β4b (Kim et al., 1998).

As mentioned above, it is well known that PLC is also regulated by PTX-sensitive $G_{i/o}$ proteins (Verghese et al., 1985; Cockcroft and Gomperts, 1985). However, efforts to demonstrate stimulation of PLC isozymes by the α subunits of these G proteins proved unsuccessful. Substantial evidence indicates that the βγ subunits of $G_{i/o}$ proteins are responsible for PLC activation. Biochemical studies of Camps et al. (1992) reported that βγ subunits from transducin or brain can activate PLC from HL60 cells, human promyelocytes and neutrophils. Blank et al. (1992) independently purified a G protein βγ complex that activated PLC from liver and brain. In addition, studies with turkey erythrocyte PLC (Boyer et al., 1992) revealed that low concentrations of βγ-subunits can reverse the stimulatory effect of activated α_{11}, whereas higher concentrations of the subunits are stimulatory. These results were substantiated by experiments that showed PLC β2 activation by βγ subunits in transfected cells (Camps et al., 1992; Katz et al., 1992). Consistent with these findings, Koch et al. (1994) observed that expression of the C-terminus of βARK (which binds to βγ subunits), in COS7 cells, blocked inositol phosphate production stimulated by α2 adrenergic and M2 muscarinic receptors, which couple to G_i, but did not affect the response to α1 adrenergic and M1 muscarinic receptors, which couple to G_q. Several

studies have shown that β2 and, possibly, β3, but not β1 and β4, isozymes are targets of βγ-subunits. Camps et al. (1992) expressed the β1 and β2 isozymes in COS1 cells and demonstrated that the β2 isozyme was more responsive to G_t βγ subunits than PLC β1. Katz et al. (1992) coexpressed the β1 and β2 isozymes together with different G protein β and γ subunits in COS7 cells and examined the inositol phosphate responses to carbachol. Transfection of β_1- and γ_1- or γ_2-subunits together with PLC β2, but not PLC β1, resulted in a marked response. In "in vitro" studies, PLC β2 is well activated by βγ subunits, while PLC β1 is only weakly responsive (Camps et al., 1992). Other reports demonstrated that, in lipid vesicles, purified PLC β3 is much more responsive to βγ subunits than PLC β2 or PLC β1 (Park et al. 1993). This effect of βγ remains however to be further characterized, as expression of PLC β3 in COS7 cells showed no response to βγ (Jiang et al., 1996b); there are probably other factors, such as substrate compartmentalization, modification, and membrane interaction and the involvement of accessory proteins, which may mediate specificity in the cellular system. Thus, the role of βγ in regulation of PLC β3 remains unclear. However, activation of PLC β3 by G βγ may occur in vivo in cells where the expression levels of PLC β3 are higher than those of the recombinant in COS7 cells or where the subcellular localization of PLC β3 or production of accessory proteins is differently regulated. Although the G proteins that

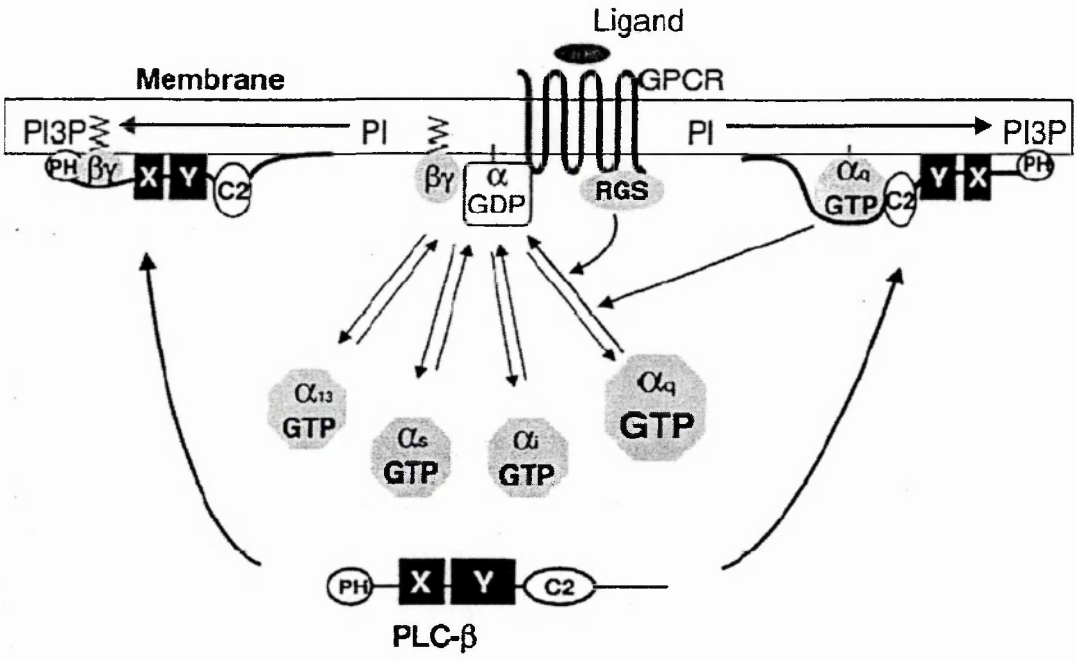


Fig. 6.GPCR-mediated activation of PLC-β isozymes. An agonist-occupied GPCR induces the exchange of GDP for GTP on the G protein α subunit and the subsequent dissociation of this subunit from the membrane-associated Gβγ dimer. In contrast to the depiction in the middle portion of the figure, the dissociated Gα subunits remain localized at the membrane as e result of their covalent lipid modification.The GTP-bound α subunits of the Gq subfamily mediate the targeting of PLC-β to the membrane and activate it as a result of interaction with the C2 domain and downstream COOH-terminal region of the enzyme (*right*). The membrane localization of PLC-β isozymes might be further promoted by interaction of the PH domain with PtdIns(3)P (PI3P) generated in response to ligation of the GPCR. Hydrolysis of GTP to GDP at the active site of Gα subunits by their intrinsic GTPase activity results in the reassociation of α with the βγ subunits and reformation of the inactive heterotrimer. RGS proteins and the COOH-terminal region of PLC-β stimulate the GTPase activity of Gqα, leading to inhibition of the GPCR signal. The membrane-anchored Gβγ dimer also recruits PLC-β by interacting with the PH and Y domains (interaction with theY domain is not shown) (*left*); this recruitment is also aided by the presence of PtdIns(3)P in the membrane. The EF-hand domain of PLC-β is not shown (Rhee, 2001).

release the activating βγ subunits are in most cases sensitive to PTX, they are not precisely identified. Different combinations of β and γ subunits have been tested for their capacity to activate PLC β (Pronin and Gautam, 1992). In one study, the functional dimers were β_1 complexed with γ_1, γ_2, γ_3, γ_5 and γ_7, and β_2 complexed with γ_2, γ_3, γ_5 and γ_7; the less effective was $\beta_1\gamma_1$ (Pronin and Gautam, 1992).

There is some evidence that agonists to receptors coupled to $G_{i/o}$ proteins can not only stimulate, but also inhibit phospholipase C (Delahunty et al., 1988; Enjalbert et al., 1990). While the PTX-sensitive stimulatory pathway has been characterized in considerable detail, the mechanism(s) and subunit(s) involved in $G_{i/o}$-mediated PLC inhibition remain to be established. Activation of dopaminergic D2 receptors on lactotrophs, which couple to both G_i and G_o (Liu et al., 1994; Watts et al., 1998), cause a PTX-sensitive inhibition of the stimulated inositol phosphate production induced by TRH (Enjalbert et al., 1986; Vallar et al., 1988; Enjalbert et al., 1990). Vallar et al. (1988) showed that dopamine progressively inhibits late inositol phosphate production induced by TRH. This inhibition appears to be a consequence of the previously demonstrated inhibition by dopamine of the prolonged cytosolic free Ca^{2+} concentration increase induced by TRH via the activation of voltage-gated Ca^{2+} channels (Malgaroli et al., 1987). Enjalbert et al. (1990) found that, in addition to the late Ca^{2+}-dependent component of the inhibitory effect of dopamine, there is also a rapid component, which might be directly

mediated by G protein subunits. In parallel, Bizzarri et al. (1990) showed that the muscarinic agonist carbachol inhibits phospholipase C via $G_{i/o}$ proteins in both intact and permeabilized rat thyroid FRTL5 cells. Subsequently, Martin et al. (1995) have found that, in coated vesicles from bovine brain, metabotropic glutamate receptor agonists inhibit guanine nucleotide-stimulated phospholipase C activity through pertussis toxin-sensitive G proteins. Interestingly, dual regulation of PLC activity by guanine nucleotides has been demonstrated in rat cerebral cortical membranes (Litosch, 1989) and mast cells (Avidor et al., 1990). Inhibition of PLC activity occurred with nanomolar guanine nucleotide concentrations, whereas stimulation ensued with micromolar concentrations of guanine nucleotides. Additional work showed that solubilized bovine brain membrane preparations retain G protein-dependent inhibition of PLC activity which occurs through a rapid but transient mechanism and results in a decrease in the Ca^{2+} sensitivity of PLC (Litosch, 1993). Taken together these data suggest the possibility that an inhibitory $G_{i/o}$ protein subunit might directly mediate receptor-induced inhibition of phospholipase C.

SPECIFIC BACKGROUND

The work presented in this thesis has investigated the possible role of the α subunits of the $G_{i/o}$ proteins in inhibition of phospholipase C activation by stimulatory receptors. The experimental approach chosen has been the expression of mutationally activated G α_i and/or G α_o subunits in Ltk^- and COS7 cells.

In the course of these studies, it has been found that in both cell types G protein-mediated stimulation of phosphoinositide hydrolysis involves a novel isoform of PLC β4 (PLC β4c). The characterization of this PLC β4 variant is also presented.

RESULTS

REGULATION OF POLYPHOSPHOINOSITIDE HYDROLYSIS BY Gαo PROTEIN IN Ltk⁻ FIBROBLASTS

Expression of $G\alpha_o$ in Ltk⁻ clones

The mechanisms of regulation of PLC by Gi/o protein α subunits have been investigated in mouse Ltk⁻ (thymidine kinase⁻) fibroblasts, a cell line already characterized in our lab for stable transfection experiments. The latter cells were stably transfected with the wild-type (WT) or a constitutively active mutant (Q205L) form of the α_o subunit. The Q205L substitution in α_o, consistent with the effects of corresponding activating mutations in α_s and α_{i2}, which inhibit the GTPase activity of the proteins (Landis et al., 1989; Freissmuth and Gilman, 1989; Wong et al., 1991), creates a mutant subunit that constitutively activates downstream effectors (Kroll et al., 1992; Wong et al., 1992). As control, I used Ltk⁻ cells that were transfected with cDNAs encoding $G\alpha_o$-WT and $G\alpha_o$-Q205L but failed to express the corresponding proteins. This effect was probably due to the integration of the transgenes in transcriptionally inactive regions of the genome or to silencing processes. Immunoblot analysis of the particulate fractions of Ltk⁻ clones confirmed the overexpression of the transfected subunits in the positive clones (lanes 3 and 5, Fig.1) when compared with the signal corresponding to the endogenous level of $G\alpha_o$in the control clones (lanes 1, Fig.1). In the same positive clones, pretreatment of the cells with Na-butyrate (lanes 4 and 6, Fig.1), enhanced the overexpression of $G\alpha_o$-WT and $G\alpha_o$-Q205L with respect to non treated

clones, while it did not affect the endogenous level of $G\alpha_o$ (lanes 2, Fig.1). The identity of $G\alpha_o$-WT and $G\alpha_o$-Q205L was confirmed by diagnostic digestion with the restriction enzyme Hae III. $G\alpha_o$ cDNA containing the Q205L mutation lacks this restriction site (data not shown).

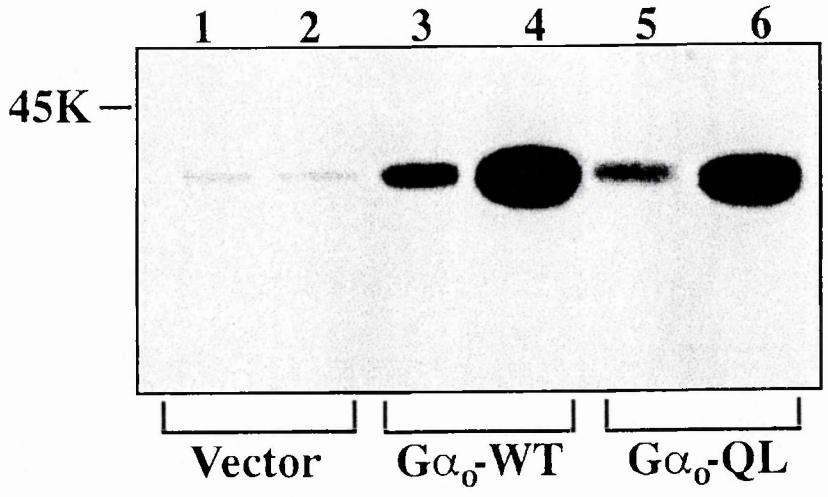


Fig.1. Analysis of the expression of $G\alpha_o$ in Ltk^- clones.

Membrane proteins (100 μg) from three representative Ltk^- clones transfected with vector or wild type (WT) or mutant (Q205L) $G\alpha_o$ were separated on a 10% SDS polyacrylamide gel and detected by immunoblotting with a $G\alpha_o$-specific antibody as described in MATERIALS AND METHODS. Cells were mantained in normal medium (lanes 1-3-5) or exposed to Na-butyrate (lanes 2-4-6) as described in MATERIALS AND METHODS. The relative molecular mass marker (in kDa) is shown to the left. Data are representative of three similar experiments.

Effect of $G\alpha_o$ on inositol phosphate production in Ltk^- cells

To investigate the effects of α_o on PLC activity, I stimulated inositol phosphate production by agonist stimulation of both PTX-insensitive (ATP) and PTX-sensitive (LPA) G protein-coupled receptors. As expected, preliminary experiments in clones of Ltk^- cells transfected with the cDNAs of wild type or mutant $G\alpha_o$ but not expressing the corresponding proteins showed that ATP-activated inositol phosphate production was insensitive to PTX, while the LPA response was inhibited by approximately 70% by PTX (Fig. 2A). Fig. 2B shows the results of a set of experiments in stably transfected Ltk^- clones, expressing the wild-type or the constitutively active mutant form of α_o. In control clones, IP release was increased 4-5 fold by ATP and 8-11 fold by LPA compared with non stimulated cells. Transfection with $G\alpha_o$-Q205L did not affect the basal inositol phosphate production, but led to a 25% reduced activation of PLC stimulated by ATP and to a 30% reduced activation of PLC stimulated by LPA compared with control cells. Expression of $G\alpha_o$-WT failed to alter basal and ATP- or LPA-stimulated inositol phosphate accumulation. From this preliminary result, I could hypothesize a possible inhibitory role of $G\alpha_o$-Q205L in the regulation of PLC activity, directly or by the involvement of an inhibitory component. Finally, I could not exclude that the observed effect was due to aspecific compensatory mechanisms resulting from the prolonged expression of the mutant protein.

To test at least partially this hypothesis, I tried to reproduce the reduction of agonist-stimulated activity of PLC upon a short-time enhancement of the overexpression of the transfected subunits, by treating the cells with Na-butyrate for 48 hours before the PI assay.

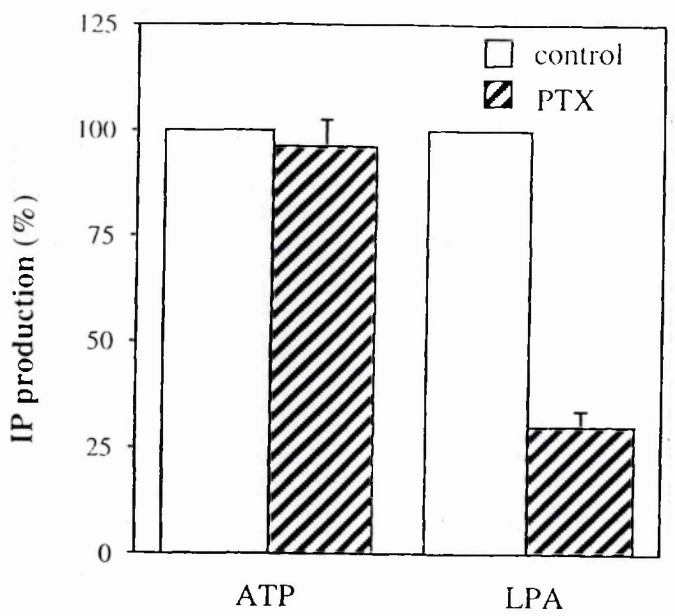


Fig. 2A. Effect of $G\alpha_o$ on inositol phosphate production in Ltk^- cells.

Clones of Ltk^- cells transfected with the cDNAs of wild type or mutant $G\alpha_o$ but not expressing the corresponding proteins were stimulated with 100 μM ATP or LPA for 30 min and treated or not with PTX. Inositol phosphate production was measured as described in MATERIALS AND METHODS. Data are means ± S.E. of the results obtained with three clones, each assayed in at least two experiments in triplicate determinations.

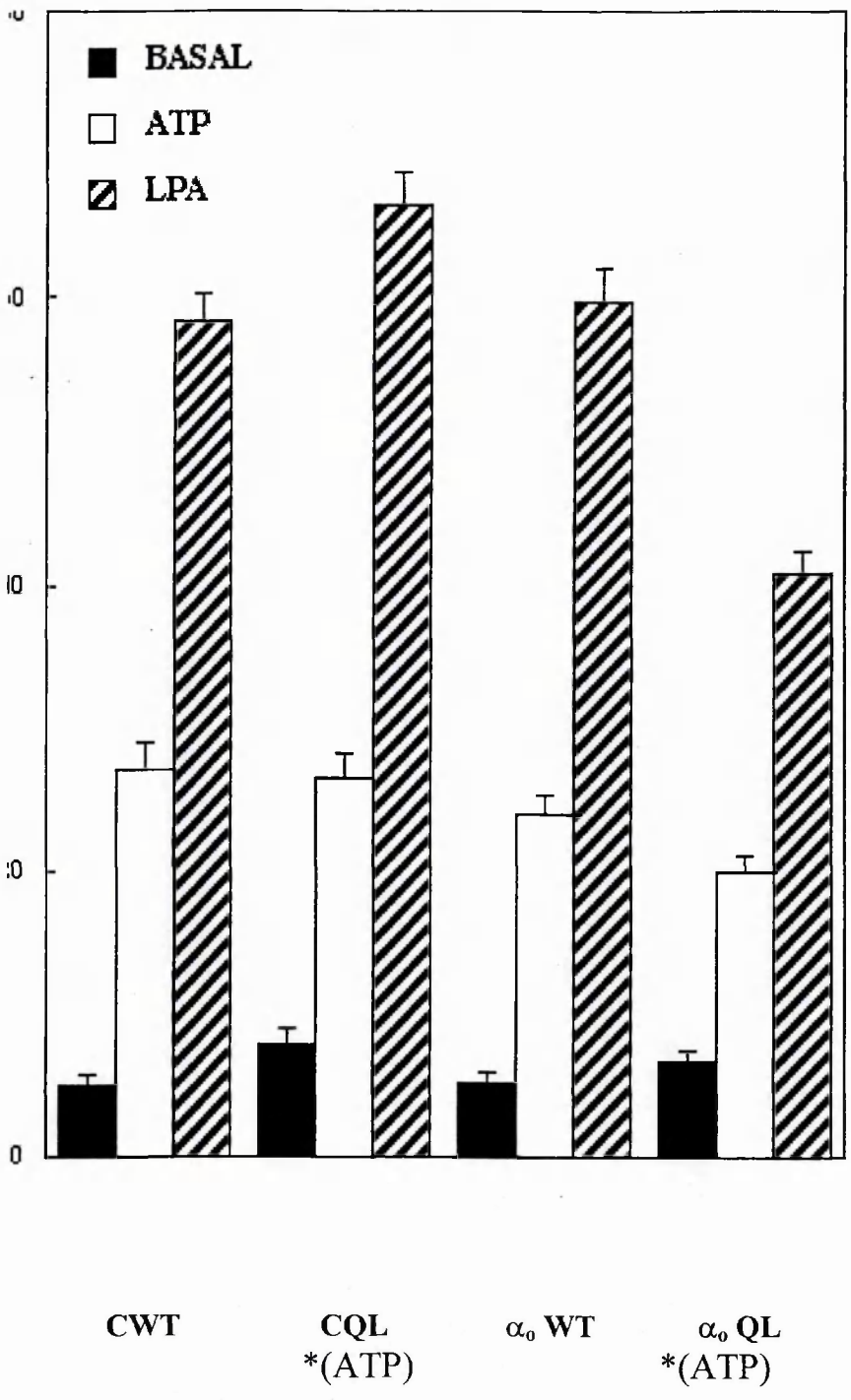


Fig. 2B.

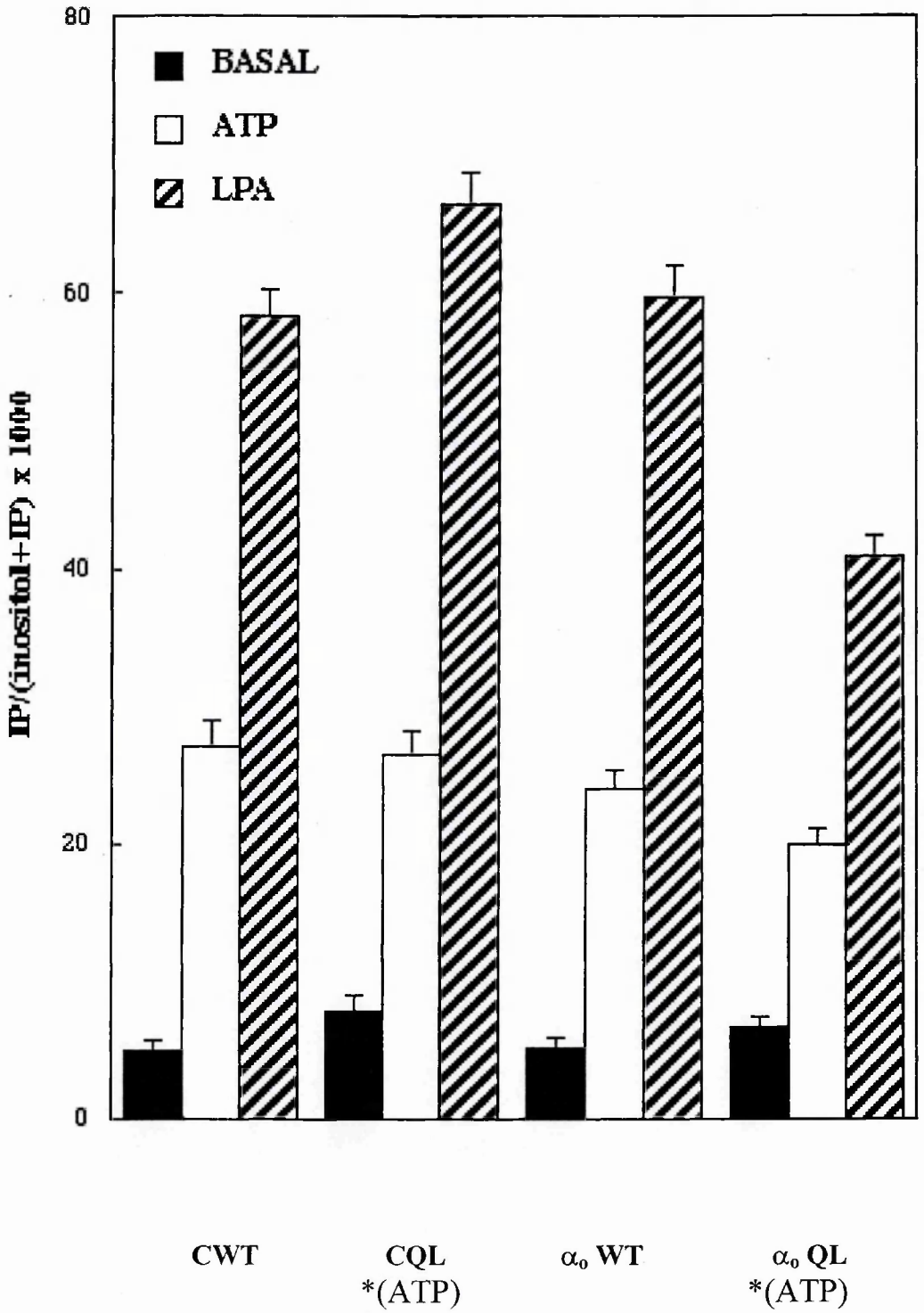
Fig. 2B. Effect of $G\alpha_o$ on inositol phosphate production in Ltk$^-$ cells.

Clones of Ltk$^-$ cells expressing wild type (WT) or mutant (QL) $G\alpha_o$ and clones transfected with the same cDNAs but not expressing the corresponding proteins (Control-WT and -QL) were stimulated with 100 μM ATP or LPA for 30 min. Inositol phosphate production was measured as described in MATERIALS AND METHODS. Data are given as ratios of the increase in IP production induced by ATP or LPA over the total of inositol and inositol phosphate products and are means ± S.E. of two experiments carried out in triplicate.

α_o **QL** *(ATP)/ **CQL***(ATP) data differences are statistically significant (T-test: $p<0.1$; $n=9$)

Effect of $G\alpha_o$ on inositol phosphate production in Ltk^- cells treated with Na-butyrate

Na-butyrate is involved in the accumulation of multiacetylated forms of histones H3 and H4 which results in an alteration of chromatine structure at the nucleosomal level and in the selective DNAse I sensitivity of transcriptionally active DNA sequences in different cell types (Vidali et al., 1978; Gavazzo et al., 1997). Since exposure of transfected cells to Na-butyrate can increase the expression of transfected proteins experiments similar to those described in Fig.2 were performed in cells pretreated with this agent. Western Blot analysis showed that Na-butyrate causes a marked increase in the expression of both wild-type and mutant $G\alpha_o$ subunits (Fig.1). As shown in Fig.3, Na-butyrate, by itself decreased basal (from 5% to 2%, at least two-fold) as well as ATP- (from 27% to17%, almost two-fold) or LPA- (from 58% to 42%) stimulated IP production in control cells compared with non-treated cells (Table 3). After treatment with Na-butyrate, the clones expressing $G\alpha_o$-WT gave results entirely comparable to those observed in control clones. In contrast, in $G\alpha_o$-Q205L-transfected clones, Na-butyrate induced an approximately two-fold enhancement of the inhibition of ATP- and LPA-stimulation.


80
60
40
20
0
BASAL
ATP
LPA
IP/(inositol+IP) x 1000
CWT
CQL
*(ATP)
α_o WT
α_o QL
*(ATP)

Stimulus	Basal		ATP		LPA	
Na-butyrate	-	+	-	+	-	+
Control-wt	4.86 ± 0.59	1.96 ±0.36	27.02 ±1.9 (5 fold)	15.73 ±1.73 (8 fold)	58.1 ±1.89 (12 fold)	42.29 ±3.6 (22 fold)
Control-QL	7.83 ±1.08	2.95 ±0.19	26.48 ±1.6 (4 fold) □	15.24 ±2.78 (5 fold)	66.2 ±2.16 (8 fold)	37.11 ±1.96 (13 fold)
$G\alpha_o$ wt	5.13 ±0.6	1.14 ±0.06	24.05 ±1.13 (5 fold)	16.88 ±0.98 (15 fold)	59.46 ±2.1 (12 fold)	36.55 ±0.95 (32 fold)
$G\alpha_o$ QL	6.75 ±0.65	1.47 ±0.08	20.01 ±1.1 (3 fold)	7.14 ±1.47 (5 fold)	40.81 ±1.51 (6 fold)	16.55 ±0.49 (11 fold)

Table 1. All data are presented as the ratio of [^{3}H]IP divided by [^{3}H] inositol plus [^{3}H] IP x1000.

Fig. 3. Effect of $G\alpha_o$ on inositol phosphate production in Ltk$^-$ cells treated with Na-butyrate.

The indicated clones of Ltk$^-$ cells were pretreated with Na-butyrate as described under MATERIALS AND METHODS. Cell stimulation and measurement of inositol phosphate production were as described in the legend to Fig. 2. Data are means ± S.E. of the results obtained with three clones, each assayed in at least two experiments in triplicate determinations. **α_o QL** *(ATP)/ **CQL** *(ATP) data differences are statistically significant (T-test: $p<0.1$; $n=9$)

Effect of $G\alpha_{i1}$ on inositol phosphate production in Ltk^- cells

To test if the effect on PLC activity observed in $G\alpha_o$-Q205L-transfected clones was specifically triggered by the expression of mutant $G\alpha_o$ and not of any other mutant $G\alpha$ subunits, parallel experiments were performed in Ltk^- clones expressing the wild-type and the active mutant form of $G\alpha_{i1}$. In contrast to the results obtained in cells expressing $G\alpha_o$, ATP- and LPA-stimulated inositol phosphate accumulation was not affected by expression of either $G\alpha_{i1}$–WT or $G\alpha_{i1}$-Q204L (Fig.4).

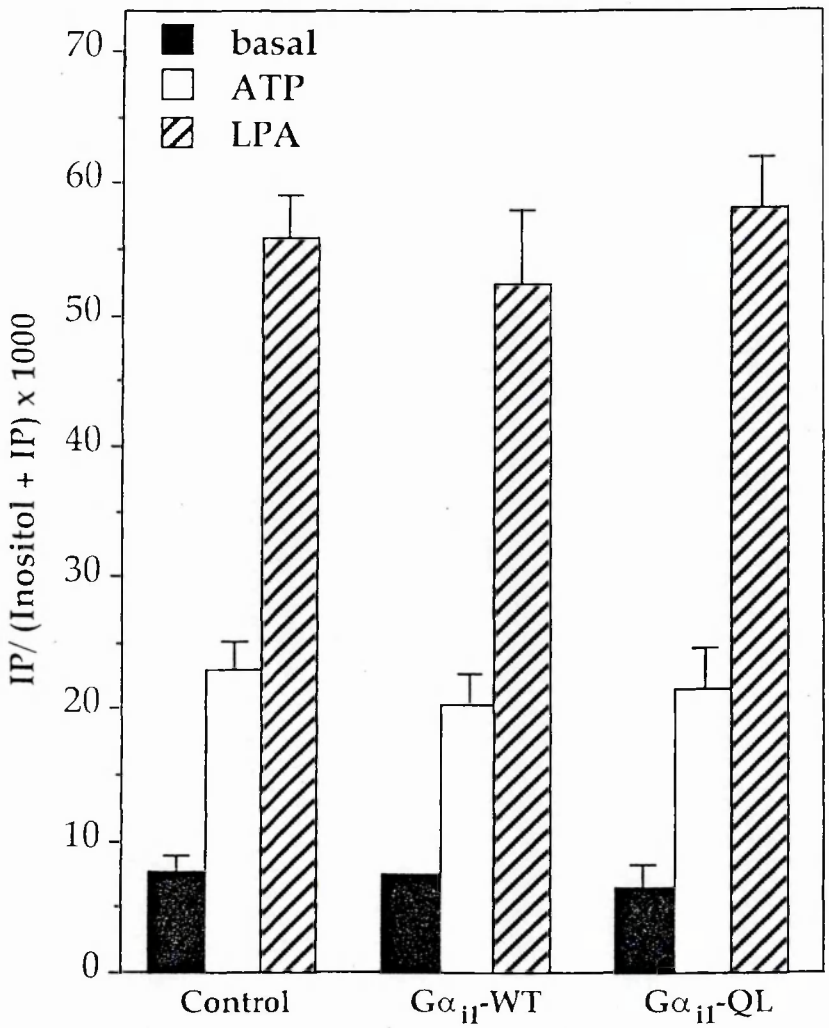


Fig. 4. Effect of $G\alpha_{i1}$ on inositol phosphate production in Ltk$^-$ cells.

Clones of Ltk$^-$ cells expressing wild type (WT) or mutant (QL) $G\alpha_{i1}$ or transfected with the latter cDNA but not expressing the corresponding protein were stimulated and assayed for inositol phosphate production as described in the legend to Fig.2. Data are means ± S.E. of two experiments carried out in triplicate on three representative clones. Similar data were obtained with at least an additional independent clone.

Effects of $G\alpha_o$ and $G\alpha_{i1}$ on cAMP levels in Ltk$^-$ cells

To investigate whether in Ltk$^-$ cells expression of mutant α_o controls other effector pathways which could account for PLC inhibition, I analyzed cAMP production. It is well established that $G\alpha_o$ protein is able to inhibit adenylyl cyclase I, which, although expressed mainly in brain, is present at lower amounts also in other cell types (Wu et al., 1992; Law et al., 1993). For these experiments, Ltk$^-$ clones were stimulated with forskolin, which directly activates the catalytic subunit of adenylyl cyclase (Premont et al., 1996). In cells expressing $G\alpha_o$-Q205L, forskolin stimulation of cAMP levels was comparable to that detected in control and $G\alpha_o$-WT clones (Fig. 5). Parallel experiments were performed by using clones transfected with $G\alpha_{i1}$, which regulates adenylyl cyclase negatively (Birnbaumer, 1990). As expected, expression of a constitutively activated α_{i1} (-QL) decreased cAMP levels. These results exclude the possibility that the effect of $G\alpha_o$ on inositol phosphate production is due to inhibition of intracellular cAMP accumulation.

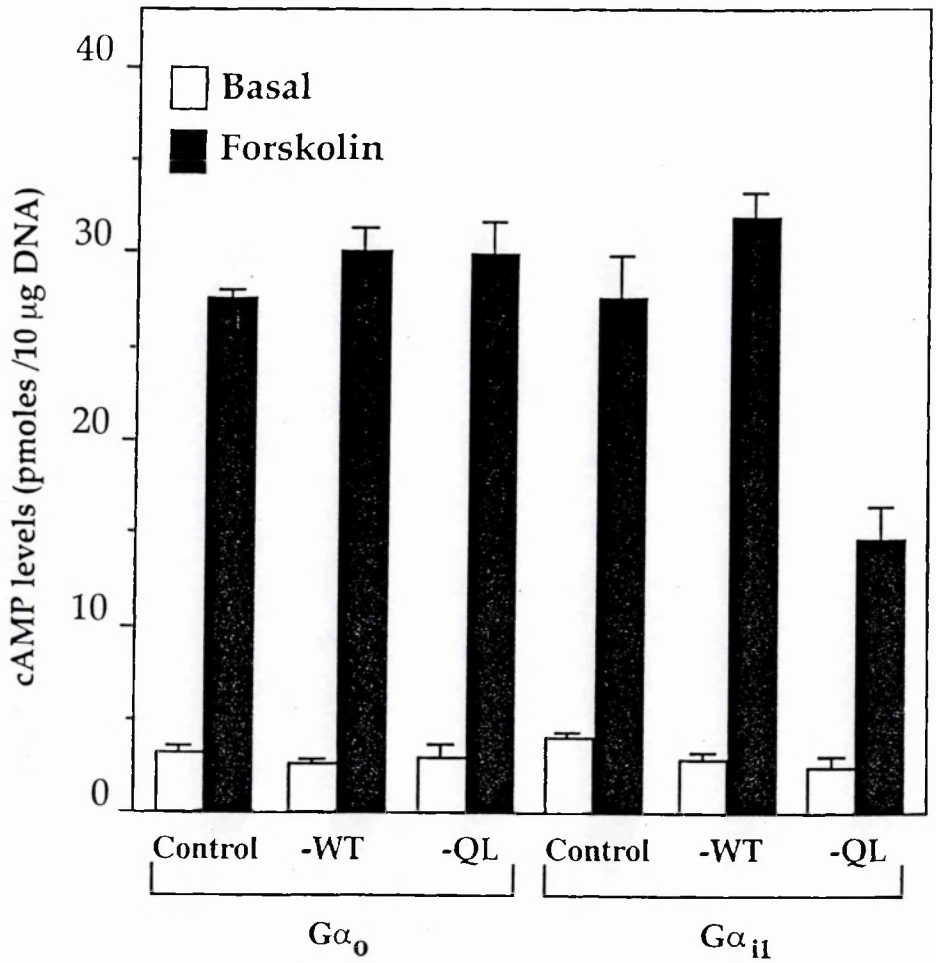
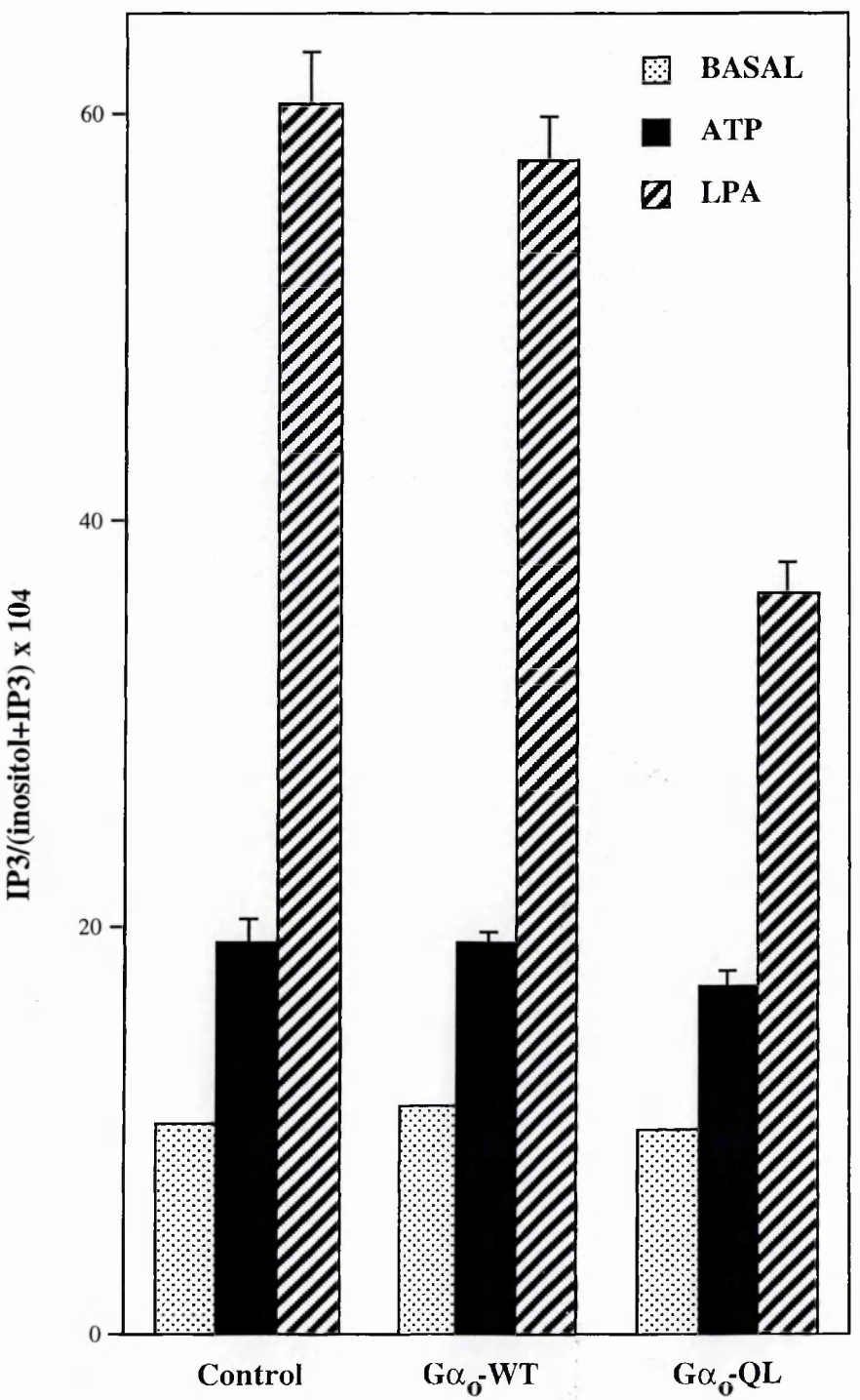


Fig. 5. Effects of $G\alpha_o$ and $G\alpha_{i1}$ on cAMP levels in Ltk⁻ cells.

The indicated clones of Ltk⁻ cells were stimulated with 20 μM Forskolin for 20 min and cAMP levels were assayed as described in MATERIALS AND METHODS. Results are means ± S.E. of two experiments carried out in triplicate on representative clones. Similar results were obtained with at least an additional independent clone.

Effect of $G\alpha_o$ on IP_3 production in Ltk^- cells incubated in Ca^{2+}-free medium

It is well established that PLC activity is dependent on intracellular $[Ca^{2+}]_i$ (Exton, 1996). In addition, it has been reported that receptor-mediated PLC inhibition is, at least partially, dependent on the inhibition of Ca^{2+} influx (Vallar et al., 1988). However, the experiment illustrated in Fig.6 shows that removal of extracellular Ca^{2+} is without effect on the ability of $G\alpha_o$-Q205L to inhibit PLC activity stimulated by ATP or LPA. Moreover, in the presence of the Ca^{2+} ionophore ionomycin, which markedly augments $[Ca^{2+}]_i$, the basal levels of inositol phosphates as well as the levels induced by the ATP or LPA stimuli and the $G\alpha_o$-Q205L inhibition remained unchanged (data not shown). This is in agreement with the observation by Bizzarri et al. (1990) that, in FRTL5, the PLC activity is not stimulated by Ca^{2+}.


Ca 2+-free medium
BASAL
ATP
LPA
IP3/(inositol+IP3) x 104
60
40
20
0
Control
Gα$_o$-WT
Gα$_o$-QL

Stimulus Ca^{2+}	Basal		ATP		LPA	
	-	+	-	+	-	+
Control	10.21 ±0.001	11.3 ±1.04	19.27 ±1.09	26.25 ±2.52	60.54 ±2.54	65.92 ±1.75
$G\alpha_o$ wt	11.2 ±0.001	11.57 ±0.9	19.25 ±0.43	25.56 ±0.73	57.74 ±2.18	59.3 ±1.6
$G\alpha_o$ QL	8.18 ±0.002	10.95 ±0.72	17.09 ±0.72	19.52 ±1.20	36.43 ±1.41	39.32 ±1.32

Table 2. All data are presented as the ratio of [^{3}H]IP divided by [^{3}H] inositol plus [^{3}H] IP x10000.

Fig. 6. Effect of $G\alpha_o$ on IP_3 production in Ltk$^-$ cells incubated in Ca^{2+}-free medium.

The indicated clones of Ltk$^-$ cells were stimulated with 100 μM ATP and LPA after incubation in Ca^{2+}-free medium as described in MATERIALS AND METHODS. IP_3 was measured as described under MATERIALS AND METHODS. Data given are means ± S.E. of the results obtained with two clones, each assayed in at least two independent experiments, carried out in triplicate.

Effect of $G\alpha_o$ on inositol phosphate production in COS7 cells

To study the possible regulation of PLC activity by $G\alpha_o$, I tried to reproduce in transiently transfected Ltk$^-$ cells the effect observed in Ltk$^-$ clones. The advantage of using this experimental approach is the possibility of cotransfecting multiple proteins in the same cells so that a signalling cascade can be reconstituted and analyzed in vivo. Unfortunately, the efficiency of transient transfection of Ltk$^-$ cells was too low to give reproducible results. I therefore tried to reconstitute the $G\alpha_o$ signalling pathway in monkey kidney fibroblast COS7 cells, which are widely used for transient transfection experiments (Conklin et al., 1992; Wu et al., 1992; Slepak et al., 1995). I first verified that $G\alpha_o$-Q205L is also able to inhibit receptor-mediated stimulation of PLC in this system. To this aim, I measured inositol phosphate production after cotransfection of the G_q-coupled α1 adrenergic receptor together with $G\alpha_o$-WT or $G\alpha_o$-Q205L and stimulation with phenylephrine. Transfection with the α1 adrenergic receptor alone, allowed phenylephrine-mediated generation of inositol phosphate release (Fig.7), but cotransfection with $G\alpha_o$-Q205L did not suppress this response (variations in inositol phosphate production are not significantly different). It should be noted that in these experiments $G\alpha_o$-WT slightly inhibited the phenylephrine response ($p<0.05$ T-test). A marked inhibition could be observed by increasing the $G\alpha_o$-WT cDNA concentration used for transfection (data not shown). A feasible

mechanism to rationalize the attenuation of the $G\alpha_q$-mediated activation of PLC by cotransfected $G\alpha_o$-WT is the competition between $G\alpha_o$ and the endogenous $G\alpha_q$ subunits for the $\beta\gamma$ complexes, which are necessary for the G protein coupling with receptors (Slepak et al., 1995). Thus, transfected wild type $G\alpha_o$ would decrease the formation of heterotrimers containing $G\alpha_q$ subunits available for $\alpha 1$ adrenergic receptor-mediated PLC activation. In contrast to $G\alpha_o$-WT, $G\alpha_o$-QL does not exert the same effect because unable to efficiently associate to $\beta\gamma$ complexes.

Since the effect of the constitutively active form of $G\alpha_o$ on PLC activation in Ltk^- clones was not reproduced in transient transfection experiments in COS7 cells, I cannot exclude that the results in Ltk^- clones were due to redundant effects resulting from the prolonged expression of the mutant protein, even if a short pretreatment of the cells with Na-butyrate enhanced the effect. A strong disregulation of the system could also result from the use of pcDNA I expression vector, which contains SV40 Ori site, for transient transfection experiments in COS7 cells, a cell line transformed by SV40.

Furthermore, the lack of induction of IP production by exogenous PLC $\beta 3$ and - $\beta 4$ in control could be explained by the lack of $G\alpha_q$ which was not overexpressed and could represent the limiting factor in this experiment. Alternatively, $G\alpha_o$ could inhibit PLC through intermediate components not available in COS7 line. Enzymes involved in the transduction cascade such as cAMP-dependent protein kinase A and protein kinase C or PI3-kinase

could play a role in the inhibition of PLC activity. The data obtained in the presence of forskolin do not support a possible role for protein kinase A, since it is shown that $G\alpha_o$-Q205L did not decrease intracellular cAMP levels. The possible role of protein kinase C should be analyzed by using its specific activator phorbol myristate acetate (PMA) or the inhibitor GF 109203X, while the involvement of PI3-kinase could be tested by using inhibitors like Wortmannin or more specifically LY 294002. More likely, I propose that $G\alpha_o$ could regulate a specific PLC enzyme expressed in Ltk^- cells and not in COS7 cells. Thus I checked for the PLC isozymes pattern expressed in Ltk^- or in COS7 cells.

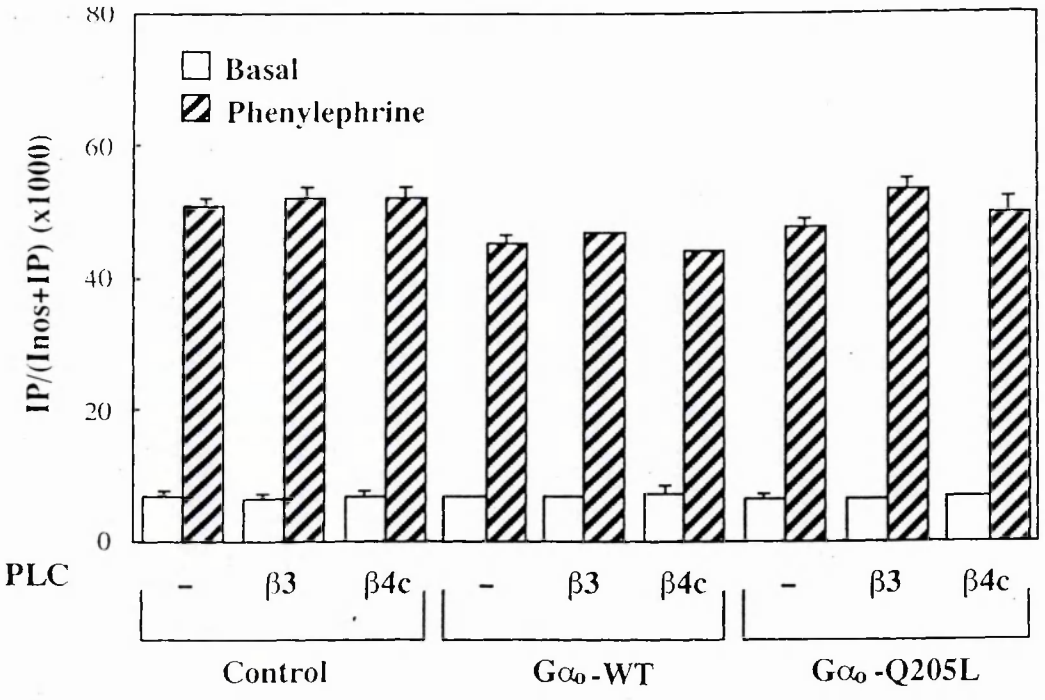


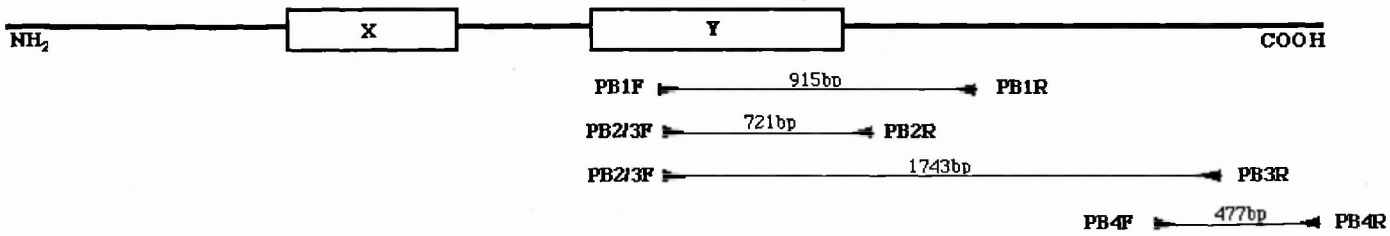
Fig. 7. Effect of $G\alpha_o$ on inositol phosphate production in COS7 cells.

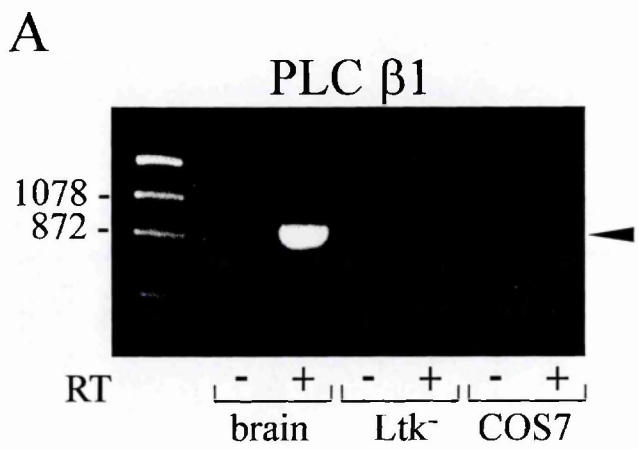
Cells were cotransfected with 3 μg of α1 adrenergic receptor and/or 5 μg of $G\alpha_o$-WT or -Q205L cDNA and/or 10 μg of PLC β3 or PLC β4c cDNA as described under MATERIALS AND METHODS. Cells were stimulated with 2 μM Phenylephrine for 30 min. Inositol phosphate production was measured as described in MATERIALS AND METHODS. Data are means ± S.E. of the results obtained in at least two experiments in triplicate determinations.

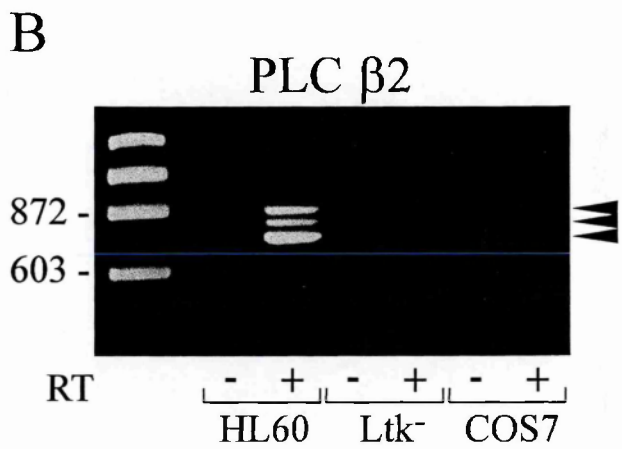
Expression of PLC β1, PLC β2, PLC β3, and PLC β4 mRNA in Ltk⁻ and COS7 cells

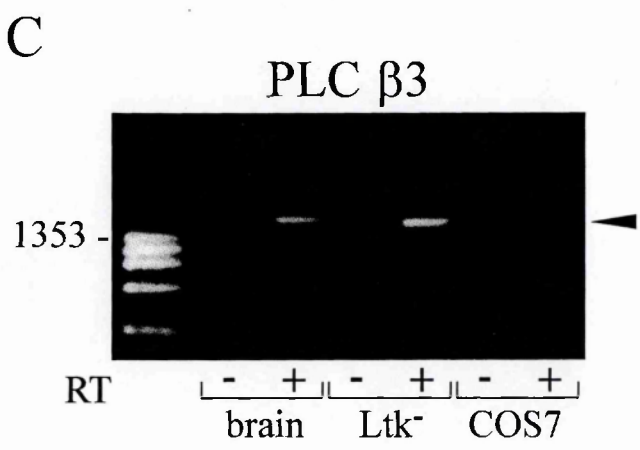
The failure to detect effects of activated $G\alpha_o$ in COS7 cells suggested, among the hypothesis explained in the former paragraph, the possibility that $G\alpha_o$ regulates a specific PLC β isozyme, expressed in Ltk⁻ cells and not or, at least not sufficiently, in COS7 cells. To test this idea, I analyzed the PLC β isozymes present in Ltk⁻ and COS7 cells (Fig.8) using RT-PCR with PLC β subtype-specific couples of oligonucleotides mapping in the C-terminal region and perfectly conserved among the species. COS7 cells are monkey cells and very little sequence information is available in that species; so, a negative result may be due to the fact that primers fail to amplify in that species. For this reason each negative result was confirmed by performing RT-PCR reaction also at very low stringency conditions (data not shown). As positive controls, brain was used in the screening for PLC β1, β3, β4 and HL60 cells for PLC β2. The identity of all the amplification fragments was controlled by diagnostic digestions (data not shown). RT-PCR for PLC β1 performed by using primers PB1F and PB1R gave the expected band of 915bp in brain, but not in Ltk⁻ or in COS7 cells (Fig. 8). RT-PCR for PLC β2 with primers PB2/3F and PB2R is shown in Panel B: the band of 721bp corresponding to PLC β1, present in HL60 cells, appears neither in Ltk⁻ nor in COS7 cells. Two additional bands were detected in HL60 cells. Diagnostic

digestion revealed that these fragments were consistent with three putative variants of PLC β2. RT-PCR for PLC β2 with different couples of oligonucleotides produced the same results (data not shown). Panel C shows the results of screening experiments for PLC β3 carried out by using primers PB2/3F and PB3R: the same expected band of 1743bp is amplified in brain as well as in Ltk$^-$ cells, but not in the COS7 line. Finally, Panel D shows the RT-PCR for PLC β4 obtained by using primers PB4F and PB4R. In the brain, two bands are detectable: the expected fragment of 477bp and another product of about 500bp present also in Ltk$^-$ and COS7 cells. As will be described in detail below, the RT-PCR product in Ltk$^-$ and COS7 cells corresponds to a novel PLC β4 variant termed PLC β4c. Thus, among the known PLC β isozymes, these results identify PLC β3 and/or PLC β4c as possible targets of $G\alpha_o$. I therefore cotransfected PLC β3 and overexpressed PLC β4c in COS7 cells together with α1 adrenergic receptor. As shown in Fig.7, in cells expressing PLC β3 and PLC β4c, cotransfection of $G\alpha_o$-Q205L had no effect on α1 adrenergic receptor-mediated increase of inositol phosphate production. These results exclude PLC β3 and PLC β4c as direct targets of α_o.


NH2
X
Y
COOH
PB1F
915bp
PB1R
PB2/3F
721bp
PB2R
PB2/3F
1743bp
PB3R
PB4F
477bp
PB4R


A
PLC β1
1078
872
RT
- + - + - +
brain
Ltk-
COS7


B
PLC β2
872
603
RT
- + - + - +
HL60
Ltk-
COS7


C
PLC β3
1353
RT
- + - + - +
brain
Ltk-
COS7

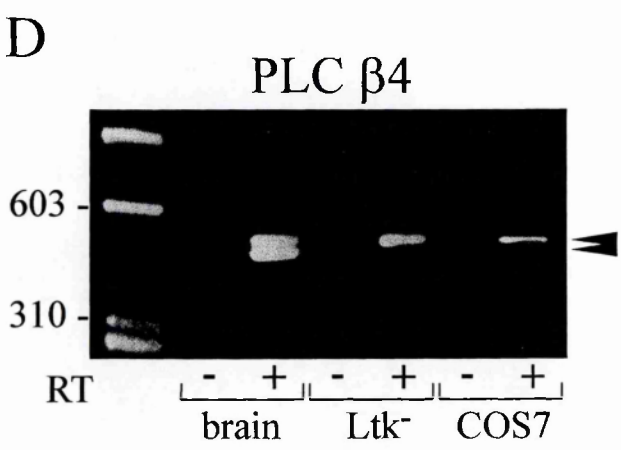

D
PLC β4
603
310
RT
- + - + - +
brain
Ltk-
COS7

Fig. 8. Expression of PLC β1, PLC β2, PLC β3, and PLC β4 mRNA in Ltk⁻ and COS7 cells.

The upper diagram shows the generic structure of PLC β and the fragments predicted to be amplified by the specific PLC β–subtype couples of oligonucleotides. RNA from the different samples was reverse transcribed using oligo(dT). PCR reactions were done with the selected primers as described in MATERIALS AND METHODS. PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Panel A: RT-PCR for PLC β1. Panel B: RT-PCR for PLC β2. Panel C: RT-PCR for PLC β3. Panel D: RT-PCR for PLC β4. In all the samples RT-PCR was performed with (RT +) or without (RT -) Reverse-Transcriptase. All the reactions performed without RNA did not amplify any fragments (data not shown). Relative sizes of DNA markers (*fX174*, Biolabs) are shown on the left of each panel. Data are representative of two independent experiments for each panel.

IDENTIFICATION OF A NEW VARIANT OF PLC β4

Diagnostic digestion of PLC β4 RT-PCR fragments

The experiments using RT-PCR carried out with primers amplifying a fragment of the C-terminal region of PLC β4a shown in Fig.8, detected in the rat brain the expected fragment of 477 bp as well as an additional product of approximately 500 bp. In mouse Ltk$^-$ and COS7 fibroblasts, the same reaction detected only the 500 bp band. The identity of the latter band was investigated by a diagnostic digestion with Bgl I-endonuclease which, as shown in Fig.9, gave a fragment of 282 bp common to the 477 bp and the 500 bp bands and other two fragments which differed of about 50 bp. These results are consistent with the hypothesis that the two amplified products corresponded to two variants of PLC β4. This result was confirmed by diagnostic digestions with other restriction enzymes (data not shown).

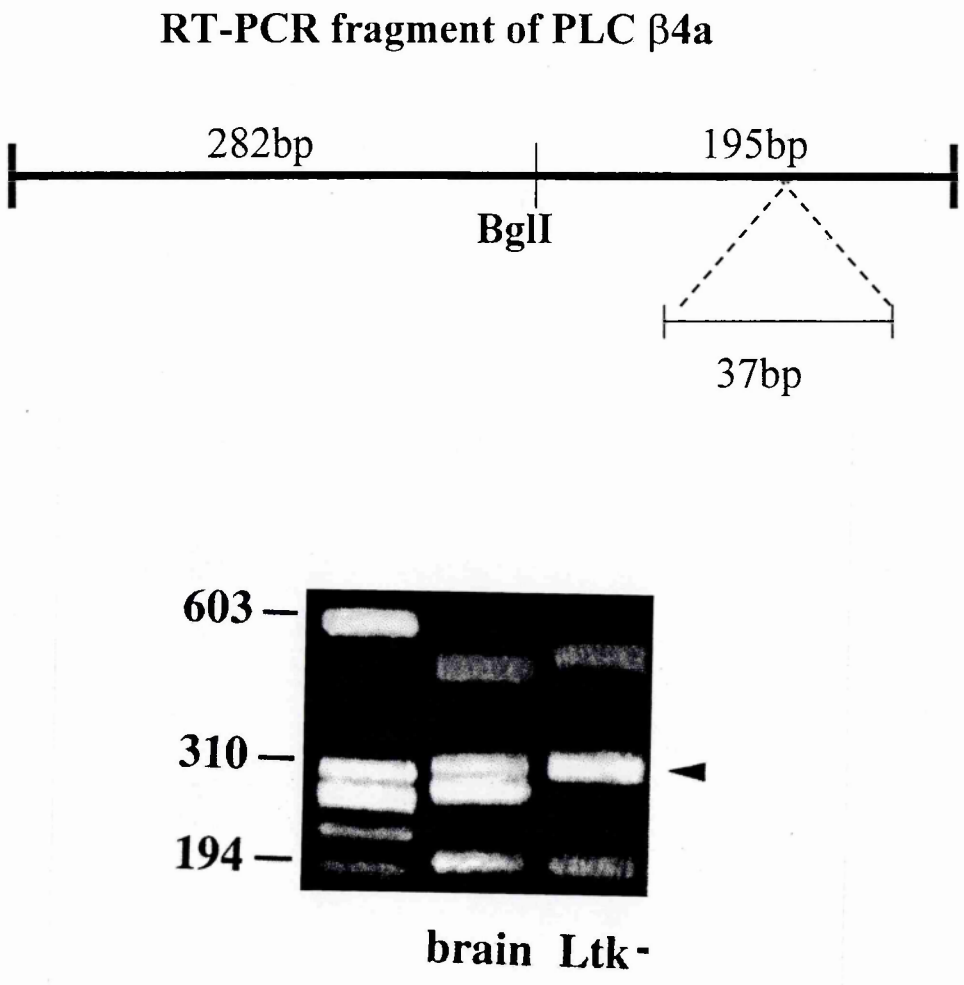


Fig. 9. Diagnostic digestion of PLC β4 RT-PCR fragments.

The amplification fragments from RT-PCR for PLC β4a were subjected to BglI digestion. Sizes of DNA marker (*fX174*, Biolabs) are shown on the left. The top band corresponds to residual undigested product. Arrowhead indicates the 282bp fragment. Data are representative of two identical experiments.

Identification of PLC β4c sequence

Further analysis of the rat brain and Ltk$^-$ cells PCR products of approximately 500 bp revealed a sequence, termed PLC β4c, identical to PLC β4a with the exception of an insertion of 37bp at 22 residues from the C-terminus. As a result, the novel PLC β4 variant exhibits a completely different 41 residue C-terminal tail (Fig.10). PLC β4 homologues from rat, bovine and human have been isolated with several exon variations (Alvarez et al. 1995; Ferreira et al., 1993). Only the two characterized rat isoforms, PLC β4a and PLC β4b, differ at their carboxyl termini (Kim et al., 1998). PLC β4a has an extensive carboxyl terminal tail, is membrane-associated and activated by $G\alpha_q$ (Alvarez et al., 1995). PLC β4c is also likely to be a membrane-associated protein and activated by $G\alpha_q$, since it contains the membrane association and $G\alpha_q$ activation domains of PLC β4a. It is possible that the carboxyl terminal tails of one PLC β isoform functions to localize this enzyme into a membrane-associated signaling complex similar to that of NORPA. Such signal transduction complexes may exist in neurons involved in visual processing. Thus I screened a rat brain cDNA library to obtain clone(s) sufficiently long to allow confirmation of the identity of the novel PLC β4 form. A 3.4Kb clone of approximately 1800bp corresponding to the coding region and

containing the extra sequence was then isolated. The N-terminal fragment of 1700bp was amplified by RT-PCR, sequenced and ligated together with the C-terminal clone; the reconstructed full-length sequence of PLC β4c was finally subcloned into the expression vector pc-DNA I.

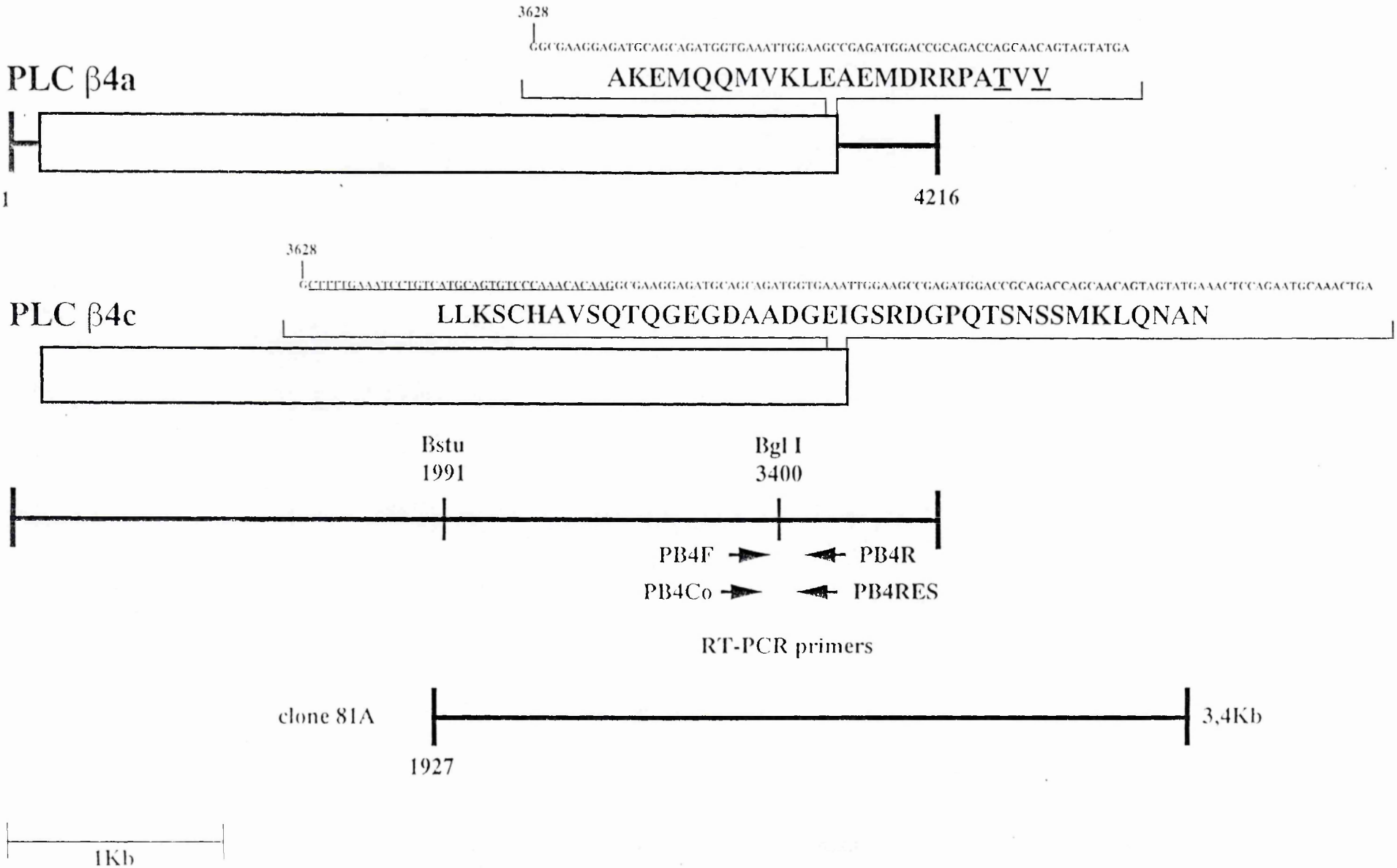

3628
GGCGAAGGAGATGCAGCAGATGGTGAAATTGGAAGCCGAGATGGACCGCAGACCAGCAACAGTAGTATGA
PLC β4a
AKEMQQMVKLEAEMDRRPATVV
1
4216
3628
GCTTTTGAAATCCTGTCATGCAGTGTCCCAAACACAAGGCGAAGGAGATGCAGCAGATGGTGAAATTGGAAGCCGAGATGGACCGCAGACCAGCAACAGTAGTATGAAACTCCAGAATGCAAACTGA
PLC β4c
LLKSCHAVSQTQGEGDAADGEIGSRDGPQTSNSSMKLQNAN
Bstu
1991
Bgl I
3400
PB4F
PB4R
PB4Co
PB4RES
RT-PCR primers
clone 81A
3,4Kb
1927
1Kb

Fig. 10. Identification of Plc β4c sequence.

Analysis of the PCR products of approximately 500 bp obtained with PLC β4 primers from rat brain and Ltk$^-$ cells was performed as described in MATERIALS AND METHODS. It revealed a sequence, termed PLC β4c, identical to PLC β4a with the exception of an insertion of 37bp. Due to the frameshift caused by this insertion, in the deduced protein sequence of PLC β4c, the C-terminal 22 residues of PLC β4a are replaced by 41 distinct amino acids. The results obtained with RT-PCR studies were confirmed by the isolation of seven independent PLC β4 clones from a rat brain cDNA library containing the 37 bp insertion. The longest clone 81A was used to reconstruct the full-length cDNA as described in MATERIALS AND METHODS.

Identification of a protein corresponding to PLC β4c

To identify a protein product corresponding to the novel cDNA, I used a synthetic peptide (CGSRDGPQTSNSSMKL) to obtain a rabbit polyclonal antibody directed against the unique C-terminal sequence of PLC β4c. Application of affinity purified preparations of this antibody in Western Blot analysis recognized a protein migrating at a rate corresponding to the expected size of 130 kDa in both rat brain and COS7 cells (Fig. 11). Expression of the 130 kDa protein was markedly increased after transfection of COS7 cells with a full-length PLC β4c cDNA. The very high level of exogenous protein is likely secondary to high-level replication of the expression vector DNA (pcDNAI) after transfection of COS7 cells, that are immortalized with an origin-defective mutant of SV40.

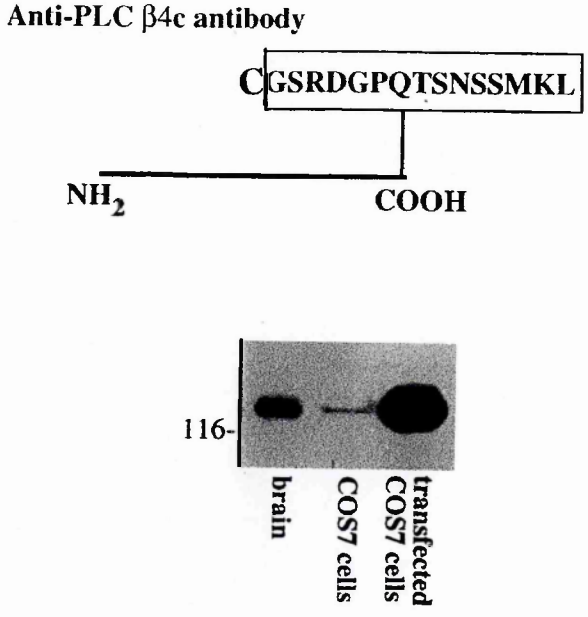
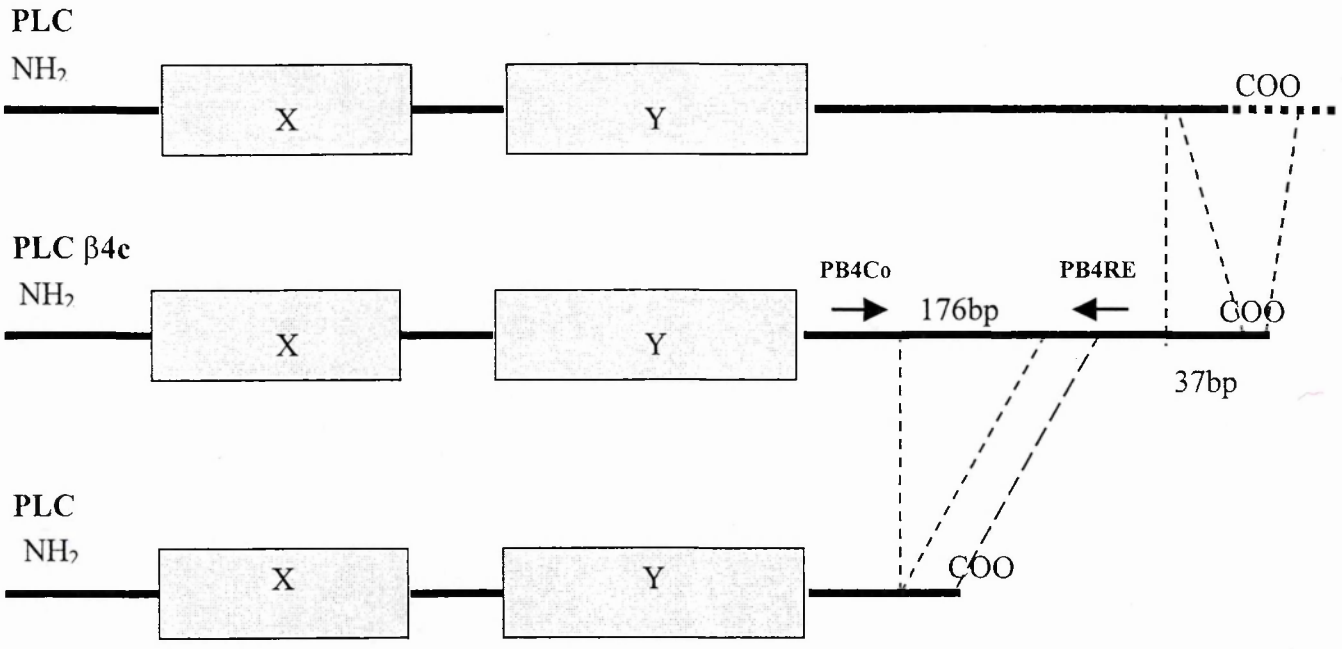


Fig. 11. Identification of a protein corresponding to PLC β4c.

A synthetic peptide represented in the upper diagram was used to obtain a rabbit polyclonal antibody directed against the C-terminal tail of PLC β4c. 200 μg of membrane fractions from brain, COS7 and transfected COS7 cells were subjected to 7% SDS-polyacrylamide gel electrophoresis and immunoblotted with affinity purified preparations of the specific antibody as described in MATERIALS AND METHODS. Data are representative of three-five similar experiments.

Screening for the expression of PLC β4b and PLC β4c

I next tested the pattern of expression of the different C-terminal variants of PLC β4. As mentioned in the Introduction, in addition to PLC β4a, a cytosolic variant has been described (PLC β4b) (Kim et al., 1998). As the cytosolic variant is mainly expressed in the cerebellum, this organ and the brain were studied by RT-PCR (Fig. 12), using two oligonucleotides that were discriminative for the deletion: the upstream primer (PB4Co) mapped at 35bp from the deletion, the downstream primer (PB4RES) primed at 271bp from the deletion in the 37bp extrasequence (see Fig. 10). The expected amplified fragments would have been 482bp for PLC β4c and 306bp for PLC β4b. By this approach, however, only the band corresponding to PLC βc was detected. Other several tissues, COS7 cells and other cell lines gave similar results (data not shown). Moreover, RT-PCR reaction was performed with other couple of oligos, but the results were consistent with the former. These findings indicate two main possibilities: the first is that the cytosolic variant does not exist and the clone isolated and characterized by Kim et al. (1998) is a simple artefact. The second possibility is that the PLC βc cDNA has a secondary structure which does not allow the annealing of the oligos used for the reactions. This last hypothesis remains unlikely, as no trace of the fragment was amplified even at low stringency conditions of amplification.


PLC
NH2
X
Y
COO
PLC β4c
NH2
PB4Co
PB4RE
176bp
37bp
PLC
NH2

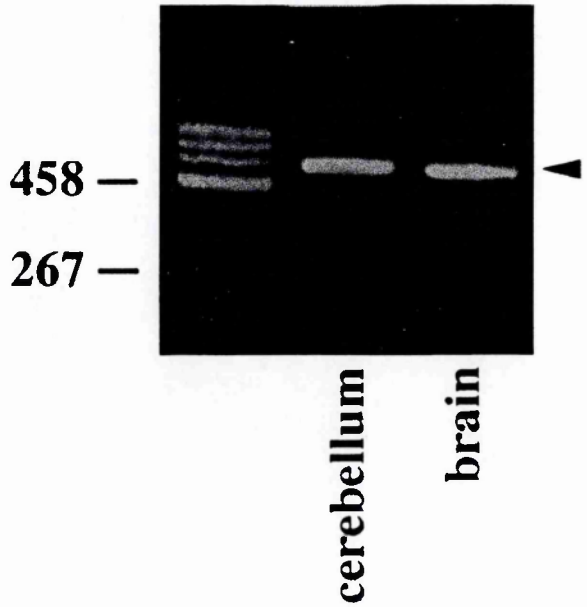

458
267
cerebellum
brain

Fig. 12. Screening for the expression of PLC β4b and PLC β4c.

RT-PCR analysis of rat cerebellum and brain mRNA was performed using primers PB4Co and PB4RES amplifying a predicted 482 bp product for PLC β4c and a 306 bp product for PLC β4b. The reaction performed without RNA or Reverse-Transcriptase did not amplified any fragments (data not shown). The 482 bp product is indicated by an arrow. Sizes of DNA markers are shown on the left. Data are representative of two independent experiments.

Expression of PLC β4a and PLC β4c by RT-PCR analysis

The patterns of expression of PLC β4a and PLC β4c were compared. As shown in Fig.13A, RT-PCR screening with primers amplifying the C-terminal region of PLC β4 (see Fig.8) showed expression of PLC β4a only in brain. In contrast, the PCR product corresponding to PLC β4c was identified in brain, COS7 and Ltk- fibroblasts and was clearly detectable also in pituitary gland, lung, kidney, spleen, thyroid FRTL5 cells, pituitary GH_4C_1 cells and chromaffin PC12 cells. The identity of the PCR fragment obtained in the various samples was confirmed by hybridization with a probe corresponding to the 37bp extrasequence (Fig. 13B). It is important to note that no PLC β4c was detected in liver and very little in spleen or COS7 cells, analogous to the expression pattern of G α_o (Price et al., 1989; Strathmann et al., 1990). This correlation of the expression patterns of PLC β4c and G α_o could reflect the existence of a pathway in which both the proteins are involved.

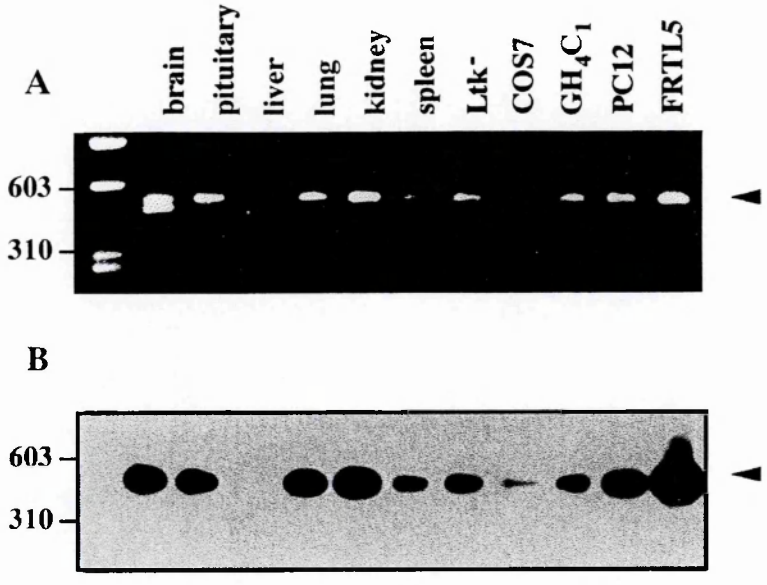


Fig. 13. Expression of PLC β4a and PLC β4c by RT-PCR analysis.

Total RNA was extracted from Sprague Dawley rat tissues and indicated cell lines and reverse-transcribed as described in MATERIALS AND METHODS. PCR (A) was performed as described in the legend to FIG 8. The data are representative of two independent experiments. The identity of the PCR fragment obtained in the various systems was confirmed by hybridization with a specific probe (B) as described in MATERIALS AND METHODS.

Western Blot analysis of the expression of PLC β4c

I tested the same samples used for RT-PCR screening and some other tissues by immunoblot analysis with PLC β4c-specific antibody (Fig. 14), as RT-PCR tecnique detects mRNA presence, but not for the effective expression of the protein. In spite of the positivity of the RT-PCR results, a clear signal in Western Blot was detectable in brain, cerebellum, lung, and all the cell lines, but not, as expected, in liver and hippocampus. A detectable signal corresponding to the protein was also absent in pituitary gland, kidney and spleen which were positive in RT-PCR screening. This was probably due to the low affinity of the specific antibody for the protein or to a lower level of expression or to an higher rate of degradation of the protein or to a combination of the above. Additional bands (immediately above and/or below the expected one) could suggest the existence of additional uncharacterized splicing variants of PLC β4c, even if they were not detected by the antibody when affinity purified (see COS7 cells lane in Fig. 11).

In summary, these results suggest that, in contrast to PLC β4a which seems mainly localized in brain, PLC β4c is more widely expressed even if in several tissues the mRNA is present but does not correspond to a comparative expression of the protein.

Tissues and cell lines	RT-PCR		Western Blot	
	PLC β4a	**PLC β4c**	**PLC β4a**	**PLC β4c**
brain	++	+	++	+
cerebellum	++	+++	+++	+++
hippocampus	not tested	not tested	-	-
pituitary	-	++	-	-
liver	-	-	-	-
lung	-	++	-	+++
kidney	-	++	-	-
spleen	-	+	-	-
heart	not tested	not tested	-	+
Ltk^-	-	+	-	++
COS7	-	+	-	+
GH_4C_1	-	+	-	+
PC12	-	++	-	+
FRTL5	-	+++	-	++

Table 3

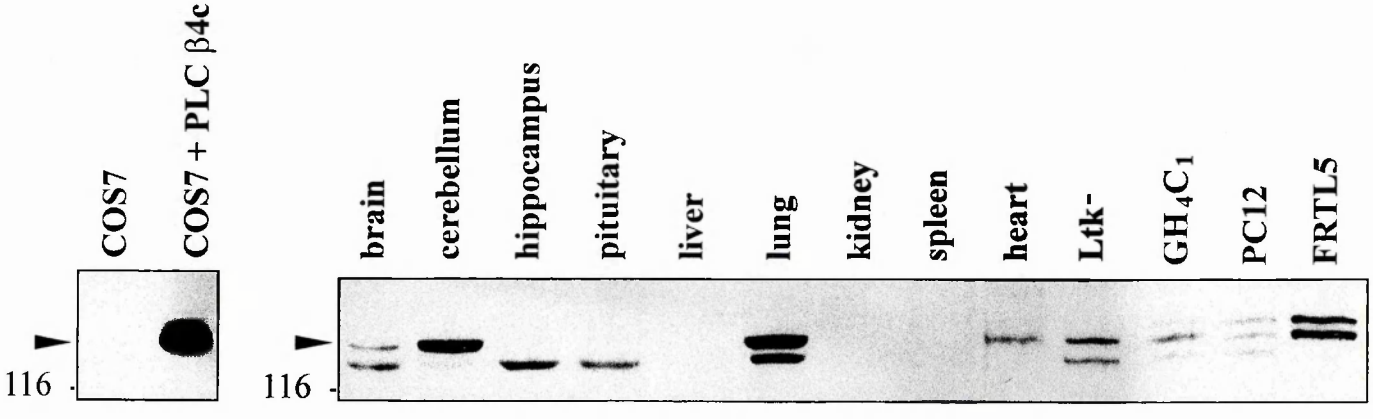


Fig. 14. Western Blot analysis of the expression of PLC β4c.

200 μg of the particulate fractions of indicated samples were immunoblotted as described in the legend to Fig. 12 and probed with anti-PLC β4c antibody affinity purified on sepharose-protein G columns (see MATERIALS AND METHODS). The bands above and below the 130 kDa band could correspond to a specific products recognized by the anti-PLC β4c antibody affinity purified on sepharose-protein G columns, as they were not detected by the same antibody purified by peptide-affinity (see Fig. 12). The relative molecular mass marker is shown to the left. Data are representative of two independent experiments.

Analysis of subcellular distribution of PLC β4a and PLC β4c

The C-terminal region of PLC β is known to be involved in membrane attachment of the enzyme (Kim et al., 1996). To assess possible functional differences between PLC β4a and PLC β4c, I therefore compared the subcellular distribution of the two enzymes. Separate analysis of soluble and particulate fractions revealed that, in agreement with previous findings, most of the PLC β4a was bound to membranes in both rat brain and transiently transfected COS7 cells (Fig. 15). PLC β4c displayed the same pattern of localization.

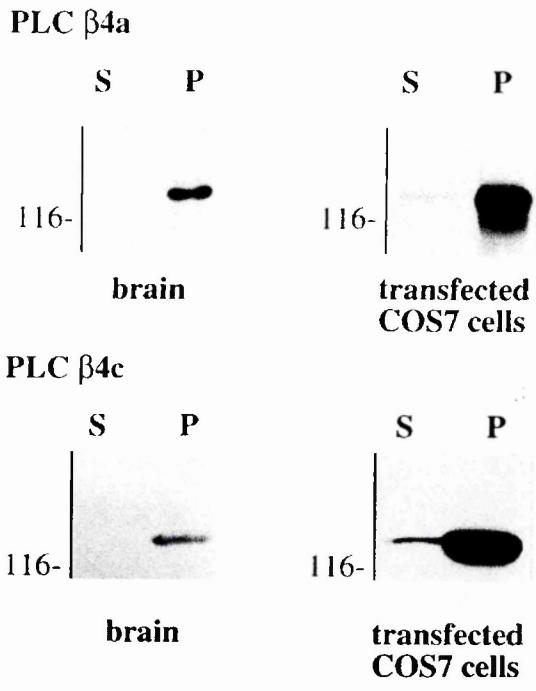
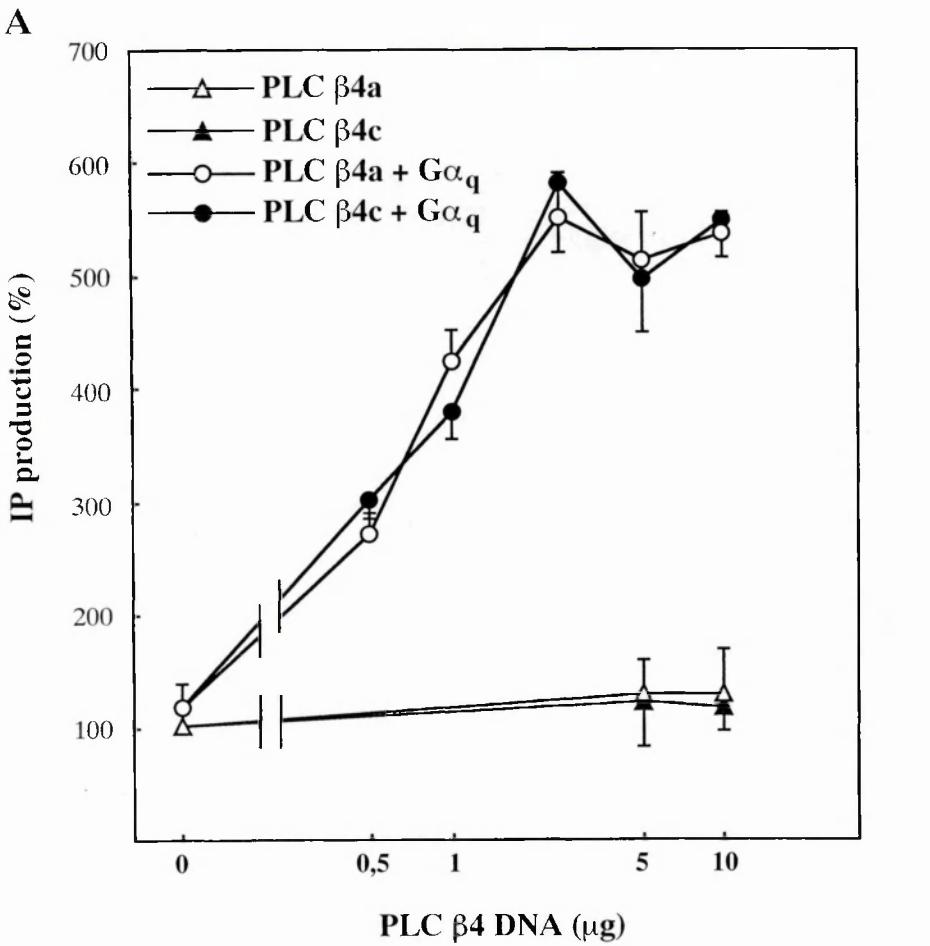


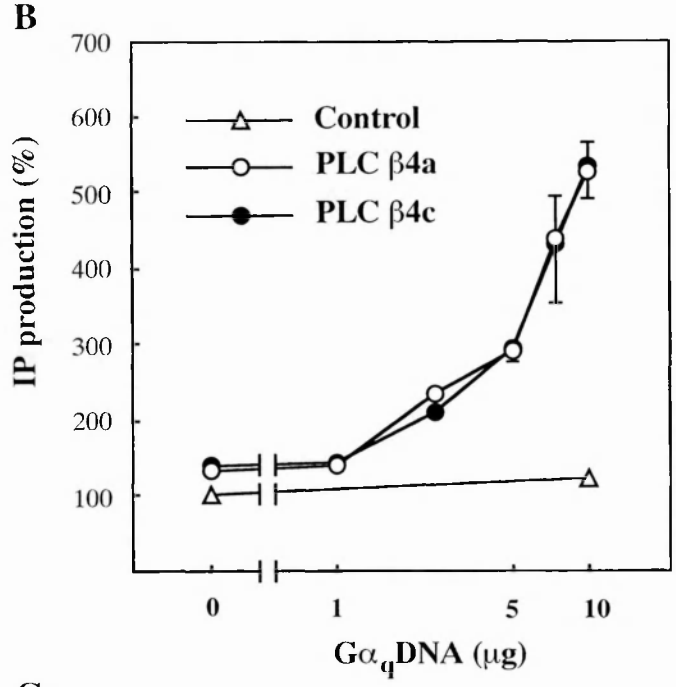
Fig. 15. Analysis of subcellular distribution of PLC β4a and PLC β4c. COS7 cells were transfected with 10 μg PLC β4a or PLC β4c as described in MATERIALS AND METHODS. Rat brain and transfected COS7 cells were fractionated as described under MATERIALS AND METHODS. 200 μg of soluble (S) or particulate (P) fractions were subjected to 7% SDS-polyacrylamide gel electrophoresis and electroblotted. The blots were incubated with anti-PLC β4a or anti-PLC β4c antibodies as described in the legend to Fig. 12. Data are representative of two independent experiments. The relative molecular mass marker is shown to the left.

PLC β4c is activated by G protein α_q and not by βγ subunits

To assess functional properties of the PLC β4a and PLC β4c variants, I investigated their pattern of regulation by $G\alpha_q$ and Gβγ subunits. Expression vectors carrying the full-length cDNAs corresponding to phospholipase C β4a and β4c were cotransfected into COS7 cells together the α subunit of G_q. As shown in Fig. 16A, transfection with vector, PLC β4a or PLC β4c leads to a low level of inositol phosphate accumulation. When $G\alpha_q$ was transfected, in agreement with previous observations (Jiang et al., 1994; Kim et al., 1998), there was an approximately 18% increase in total inositol phosphate formation compared with vector alone. When the cells were cotransfected with $G\alpha_q$ and increasing concentrations of PLC β4a or PLC β4c, inositol phosphate formation progressively increased, with both the variants of PLC β4 equally responsive to the α subunit. To exclude the possibility that these results could be due to a differential expression of PLC β4 or $G\alpha_q$ in transfected cells, we performed an immunoblot analysis using specific antibodies. These experiments confirmed that the amounts of PLC β4a and PLC β4c correlated with the increasing concentrations of transfected vectors and that the expression levels of both PLC β4 and $G\alpha_q$ were not affected by cotransfection. To test whether the two variants differ in their sensitivity to $G\alpha_q$, COS7 cells were cotransfected with the maximal concentration of PLC β4a or PLC β4c and increasing concentrations of $G\alpha_q$. As shown in

Fig.16/B, the two curves of response to $G\alpha_q$ were super-imposable for the two enzymes. These results show that the two alternative C-terminus of the two variants have no effect on interaction with $G\alpha_q$, consistently with the identification of the region deputated to this function just upstream of the C-terminus of PLC β4 protein (Wu et al., 1993). Finally, I tested the activation of PLC β4a or PLC β4c by $G\beta_1\gamma_2$ and $G\beta_2\gamma_2$ subunits. These combinations have been shown to be the most effective in PLC activation (Hawes et al., 1995). As shown in Fig.16C, $G\beta_1\gamma_2$ and $G\beta_2\gamma_2$ induced per se an approximately 100% and 40% increase of IP production, respectively, which is the effect of endogenous PLC stimulation by the excess of free βγ complexes. In agreement with previous findings (Jiang et al., 1994), expression of these subunits did not induce a significant stimulation of PLC β4a. Experiments with PLC β4c revealed that also this variant was unresponsive to $G\beta_1\gamma_2$ and $G\beta_2\gamma_2$. On the contrary, co-transfection of $G\beta_1\gamma_2$ and $G\beta_2\gamma_2$ with PLC β4 seems to diminish the effect of βγ. This effect could be due to a sort of desensitization of endogenous PLC activity by the overexpressed PLC β4 variants, which could create a loop of negative feedback on the basal PLC enzymes. Taken all together, these results suggest that both PLC β4a and PLC β4c participate in phosphoinositide hydrolysis by G protein-coupled receptors via $G\alpha_q$ and not via Gβγ subunits.


A
PLC β4a
PLC β4c
PLC β4a + Gαq
PLC β4c + Gαq
IP production (%)
PLC β4 DNA (μg)
PLC β4a
PLC β4c
DNA (μg)
anti-PLC β4
anti-Gαq
+ Gαq
116
45


B
Control
PLC β4a
PLC β4c
IP production (%)
GαqDNA (μg)

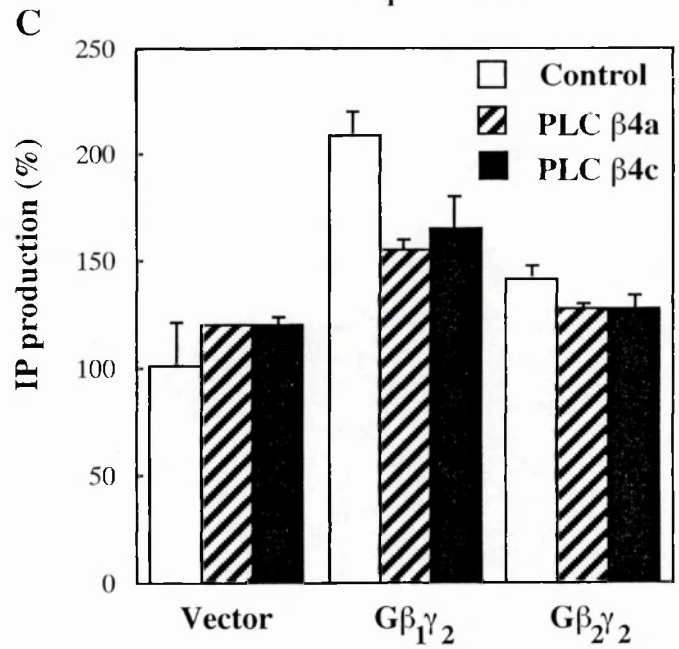

C
Control
PLC β4a
PLC β4c
IP production (%)
Vector
Gβ1γ2
Gβ2γ2

Fig. 16. PLC β4c is activated by G protein α_q and not by βγ subunits.

A: activation of PLC β4a and PLC β4c by $G\alpha_q$. Cells were transfected with 5 μg of $G\alpha_q$ vector and the indicated doses of PLC β4a and PLC β4c cDNA as described under MATERIALS AND METHODS. The level of total inositol phosphates was determined 48 hrs after transfection (see MATERIALS AND METHODS). Data are presented as percentage of IP accumulation in cells transfected with pcDNAI alone. The data shown are the means ± S.E. of three independent experiments performed in triplicate. The blots show expression of $G\alpha_q$ and the two forms of PLC β4 in COS7 cells transfected with the indicated cDNAs. The relative molecular mass markers (in kDa) are shown to the left. B: dose-dependence of the activation of PLC β4a and PLC β4c by $G\alpha_q$. Cells were transfected with 5 μg of PLC β4a or PLC β4c and increasing doses of $G\alpha_q$ vector. The experiments were performed as described for Panel A. Data shown are means ± S.E. of two-three independent experiments in triplicate determinations. C: effect of G-protein $\beta_1\gamma_2$ and $\beta_2\gamma_2$ complexes on PLC β4a and PLC β4c. COS7 cells were transfected with 5 μg of β_1, β_2, γ_2, PLC β4a and PLC β4c. The experiments were performed as described for Panel A. Given values are means ± S.E. of two experiments in triplicate determinations ($n=9$; $p<0.05$ T-test).

Effect of G α_o on polyphosphoinositide hydrolysis

Several studies have shown that various receptors coupled to PTX-sensitive G proteins inhibit polyphosphoinositide hydrolysis. However, the evidence concerning the specific G protein subunit(s) involved in these responses is still incomplete.

The experiments presented here demonstrate that the constitutively active form of $G\alpha_o$, when stably expressed in Ltk$^-$ cells, inhibits agonist-induced stimulation of inositol phosphate production. Since a similar effect was not observed in transient transfection experiments carried out with COS7 cells, one cannot exclude the possibility that the results obtained in Ltk$^-$ clones be due to compensatory mechanisms or irrelevant effects resulting from the prolonged expression of the mutant protein. However, the fact that a relatively short treatment of Ltk$^-$ cells with Na-butyrate, which increases the levels of expression of the transfected protein, markedly enhanced inhibition of inositol phosphate production by $G\alpha_o$, certainly supports the idea of an inhibitory influence of α_o.

The effect of $G\alpha_o$ on inositol phosphate production can be accounted for by the regulation at the level of all components involved in this response: these include the stimulatory receptors, the stimulatory G protein subunits and the

PLC β enzymes. In addition, there is the possibility that $G\alpha_o$ might inhibit stimulation of inositol phosphate production by affecting the synthesis or metabolism of membrane phospholipids. To investigate the target of $G\alpha_o$, I identified by RT-PCR analysis the PLC β subtypes present in Ltk^- cells. These studies revealed the expression of both PLC β3 and PLC β4c. However, in COS7 cells transfected with these enzymes, upon stimulation of the α1-adrenergic receptor, which can couple to all the α subunits of the G_q family (Wu et al., 1992), $G\alpha_o$ failed to inhibit IP production. Possible explanations of these negative results include the fact that $G\alpha_o$ acts on an unknown PLC β, present in Ltk^- but not in COS7 cells, or inhibits PLC β3 and/or PLC β4c through additional component(s) which are not available in the COS7 line. Thus, among the PLC β enzymes, PLC β3, PLC β4 or another unknown PLC β subtype remain the possible targets of $G\alpha_o$ in the Ltk^- cell model.

The component of the phosphoinositide hydrolysis signalling system sensitive to $G\alpha_o$ could be regulated by this subunit either directly or via other effector(s) and intracellular pathway(s). The results obtained in Ltk^- cells seem to exclude both the inhibition of adenylate cyclase and suppression of Ca^{2+} influx, as mediators of the effect of $G\alpha_o$ in Ltk^- cells. However, many other signals involving PKC, PI3K, Src kinases and MAPK, all of which have been recently shown to be activated by $G\alpha_o$ (Kroll et al., 1991; van

Biesen et al., 1996; Ram et al., 1999), could possibly affect the PLC signalling system. Further experiments are clearly needed to elucidate the target(s) and the mechanism(s) of action involved in $G\alpha_o$ regulation of phosphoinositide hydrolysis.

In conclusion, my findings indicate that expression of constitutively active $G\alpha_o$ can inhibit the cellular response to receptors stimulating phosphoinositide hydrolysis. The results obtained in COS7 cells suggest that this effect of $G\alpha_o$ occurs only in specific cell types. Interestingly, a genetic study in the nervous system of *C. elegans* has shown that the $G\alpha_o$ homologue, GOA-1, negatively regulates the $G\alpha_q$ homologue, EGL30, pathway, which activates the PLC β4 homologue, EGL8 (Miller et al., 1999). It seems therefore quite possible that a $G\alpha_o$-mediated signal represents at least one of the events responsible for the ability of $G_{i/o}$-coupled receptors to inhibit phosphoinositide hydrolysis in certain cellular systems.

In the last two years, several studies have characterized different signalling pathways in which $G\alpha_o$ is involved, but not concerning PLC activity. In particular, Ram et al. (2000) have shown that the expression of mutant $G\alpha_o$ Q205L in NIH-3T3 cells results in transformation through the activation of the Stat3 signaling pathway. A different study by Dhingra et al. (2000, 2002) has revealed that $G\alpha_o$ 1 variant is required by retinal ON bipolar cells for the response to rod and cone input: glutamate released into retinal ON bipolar

neurons binds to a metabotropic receptor to activate $G\alpha_o$ that ultimately closes a nonspecific cation channel. Signaling requires the $G\alpha_o$, but its effector is unknown.

In rat hippocampal synapses, $G\alpha_o$ seems to be involved together with other PTX-sensitive $G\alpha$ subunits in presynaptic inhibition triggered through the interaction with specific GPCRs (Straiker et al., 2002). To date, there is no evidence in the literature on the a regulation of the PLC signaling pathway by $G\alpha_o$. Thus, the results contained in this thesis remain to be clarified.

The PLC β4c enzyme

The PLC β family of enzymes is involved in G protein-dependent signalling pathways. PLC β subtypes varying in both the N-terminal and in the C-terminal regions have been characterized. The results of PCR amplification and rat brain cDNA library screening reported here indicate the existence of a novel C-terminal variant of PLC β4, named PLC β4c. The same sequence has been recently described by Adamski et al. (1999). However, no characterization of the distribution and functional properties of PLC β4c was performed in the latter study. The 1880 bp-long clone encoding PLC β4c that we have analyzed shares its sequence with the previously identified PLC β4a enzyme, with the insertion of an additional 37 bp of C-terminal sequence. Although it cannot be ruled out that PLC β4a and PLC β4c are the product of different genes, it appears quite likely that the two mRNAs are generated by

alternative mRNA processing. This possibility is supported by the previous identification of splicing variants of several PLCβ enzymes including rat PLC-β1 (Bahk et al., 1994), bovine PLC-β4 (Ferreira et al., 1993), *Drosophila* PLC-p21 (Shortridge et al., 1991), and *Drosophila* norpA PLC (Kim S. et al., 1995). The splicing in all of these PLC isozymes occurs outside the X and Y catalytic domains. Importantly, alternative splicing in the C-terminal regions of the rat PLC β1 and the *Drosophila* PLC-p21 has been reported (Bahk et al., 1994; Shortridge et al., 1991).

Characterization of the expression patterns of PLC β4 has only been reported for the rat brain. Results obtained by RNA hybridization of tissue sections and immunodetection by using an antibody raised against the N-terminal region of PLC β4 (common to PLC β4a, -b and -c) indicate that its expression is highest in the cerebellum and almost negligible in the cerebral cortex and hippocampus (Tanaka et al., 1994; Adamski et al., 1999). My RT-PCR experiments detected PLC β4a in the brain, in agreement with previous findings, but not in other organs. In contrast, PLC β4c mRNA is present in all tested tissues, except the liver, and cell lines. Immunoblot analysis detected a significant signal for PLC β4c only in brain, cerebellum, lung, heart, COS7, GH4, FRTL5, Ltk$^-$ and PC12 cells with the highest levels in the cerebellum. The absence of a band corresponding to PLC β4c in several of the tissues examined by Western Blotting could be explained by very low levels of

enzyme expression that are below the threshold of detection by our antibody, or to a major rate of degradation of the enzyme. Despite this limitation, the data indicate that PLC β4a is mainly, if not exclusively, present in the brain, whereas PLC β4c has a more widespread distribution.

The different patterns of expression suggest that the two variants could have different functions. The novel cDNA for PLC β4c has an insertion of 37bp that results in the replacement of the 22 C-terminal amino acids of PLC β4a with 41 different amino acids. Previous studies have identified the C-terminal region of PLC β enzymes as responsible for association to the particulate fraction (Kim et al., 1996) and activation by $G\alpha_q$ (Wu et al., 1993). By creating a series of deletion mutants, Wu et al. (1993) found that the region between residues Thr-903 and Gln-1030 (P-Box) is necessary both for association of PLC β1 with the particulate fraction and its activation by $G\alpha_q$. This report also showed that the region between residues Lys-1031 and Leu-1142 (G-box) is required for interaction with the G protein α_q subunit but is not necessary for association of PLC β1 with the particulate fraction. The P-box in PLC β1 is the most lysine-rich region in the entire molecule. It was therefore suggested that association with the particulate fraction might occur through phospholipid or some intermediate negatively charged membrane-bound proteins. Other studies have demonstrated the importance of the C-terminal basic residues of PLC β1 as well as PLC β2 for membrane

association (Park et al., 1993; Lee et al., 1993; Kim et al., 1996). I found that both variants of PLC β4 are mainly localized in membranes and are sensitive to $G\alpha_q$. These results are consistent with the above discussed studies identifying the region required for membrane association and interaction with $G\alpha_q$ upstream to the C-terminal end of the protein (Wu et al., 1993).

Kuang et al. (1996) proposed that the Glu-435 to Val-641 region of the PLC β2 molecule is involved in the interaction with the G protein βγ subunits. Binding assays using glutathione S-transferase-fused PLC β2 and pure G protein βγ subunits further narrowed the region down to 62 amino acids (residues Leu-580 to Val-641). These residues of PLC β2 are located inside the catalytic Y-domain, highly conserved among PLC isozymes. Although it has been previously shown that PLC β4a cannot be activated by G protein βγ subunits (neither in vitro nor in vivo), and the analysis of the two variant sequences tended to exclude a different regulation of PLC β4c, I checked this possibility in transfected COS7 cells. As expected, in my experimental system, PLC β4c proved to be unresponsive to two different combinations of Gβγ.

It should be mentioned that Kim et al. (1998) identified a variant of PLC β4, PLC β4b, which appears to be localized in the cytosolic fraction and to be insensitive to $G\alpha_q$. These authors isolated two PLC β4b clones but selected for further analysis only the one containing the longer cDNA insert. The

overall cDNA structure of this clone was almost identical to the PLC β4a mRNA except for a 176 bp deletion in the C-terminal region of the protein. As a result, the C-terminal 162 amino acids of PLC β4a are replaced by 10 distinct amino acids in PLC β4b. Western Blot analysis with an antibody directed against the N-terminal portion of PLC β4 and an anti-PLC β4b specific antibody, identified the truncated variant in the brain cytosol. Further immunocytochemistry studies, not shown in the report, apparently revealed high levels of PLC β4b in the cytoplasm of Purkinje cells of rat cerebellum. Using diagnostic RT-PCR, I tested brain, cerebellum and various tissues and cell lines for the presence of PLC β4b, with negative results. Clearly, further studies will be necessary to clarify this issue.

Although my experiments did not reveal any functional differences between PLC β4a and PLC β4c, there is an interesting feature that distinguishes the cDNA sequences of the two variants. Only PLC-β4a contains a C-terminal consensus sequence (Thr-Val-Val-COO-) for the PDZ domain-binding site (see Fig.10). PDZ domains are multifunctional protein interaction motifs which provide a framework for the recruitment of target molecules into membrane-bound macromolecular complexes. These domains bind to specific sequences at the C-terminus of the interacting proteins. Most interestingly, the Drosophila PLC, norpA, is an integral part of a signal transduction complex organized by a PDZ protein, INAD, which binds and

clusters protein kinase C, the TRP calcium channel, and norpA in a complex associated with the rhabdomere membrane. Recent results (van Huizen et al., 1998) have shown that Drosophila INAD, which contains five tandem PDZ domains, directly binds norpA via two terminally positioned PDZ1 and PDZ5 domains. PDZ1 binds to the C-terminus of norpA, while PDZ5 binds to an internal region overlapping with the G box-homology region. The norpA proteins lacking binding sites, which display normal basal PLC activity, can no longer associate with INAD in vivo. These truncations cause significant reduction of norpA protein expression in rhabdomeres and severe defects in phototransduction. The vertebrate PLC β4a shares greater similarity with norpA than with other PLC β isotypes (Kim et al., 1993; Lee et al., 1993). This suggests that regulation of PLC β4a-mediated signal transduction may be similar to the norpA-mediated mechanism involved in visual transduction in *Drosophila*. It is therefore possible that the presence of a putative PDZ-binding region in PLC β4a, which is expressed in the brain, and not in the more ubiquitous PLC β4c, determines the selective involvement of PLC β4a in specific signal transduction pathways operating in neurons (Jang et al., 1996; Kim et al., 1997). Besides, potential phosphorylation sites can be identified in PLCβ4c C–terminal tail (serine residues) which could be involved in specific pathways of regulation of PLC; indeed the PKC-dependent phosphorylation of PLC β3 isozyme was shown to be involved in

desensitization of PAF- (Ali H. et al., 1997) and opioid receptor-mediated (Strassheim et al., 1998) phosphoinositide hydrolysis and Ca^{2+} mobilization.

In conclusion, this thesis has shown the existence of:

-a novel mechanism of inhibitory regulation of G protein-coupled receptor-mediated activation of PLC is triggered by activated $G\alpha_o$ subunit in a specific cell type, Ltk^- cells. The pathway and the proteins involved remain to be characterized. To this aim:

-the possible involvement of intermediate messengers as src-like kinases, PKC, Rap1, PI4K, MAPK should be tested by using specific inhibitors or activators.

-transient transfection experiments should be performed in another mouse cell line than COS7 cells, which is not transformed by SV40 and expressing endogenous $G\alpha_o$ subunit.

-a novel C-terminal variant of PLC β4, PLC β4c. This enzyme is widely distributed, in contrast to the known variant PLC β4a, which is expressed only in brain and contains a putative PDZ domain-binding sequence, probably involved in the formation of neuronal specific multiprotein complexes. On the contrary, PLCβ4c does contain three putative phosphorylation sites in its specific C-teminal sequence which are absent in PLC β4a: Ser^{1218}, Thr^{1239} and Ser^{1243}. These sites could represent targets for regulation by Ser/Thr kinases, analogously to PLCβ3 (Xia et al., 2001); in

particular, Ser^{1243} is a putative phopshorylation site for PKC. Both the variants are equally responsive to G protein subunits; functional differences between the two isoenzymes have to be investigated. To this aim, several issues could be analyzed:

-characterization of both the variants expression at different stages of embryonic development and in adult tissues by in situ hybridization and immunocytochemistry (technical problems due to the low affinity of PLC β4c antibody could be bypassed by tag-labeling).

-identification of PDZ domain containing proteins binding to PLC β4a in retina (like INAD for NORPA) or cerebellum (like Homer which couples to mGluR) by two-yeast-hybrid system or immunoprecipitation assays

-characterization the function of the two variants C-terminal tails by site-directed mutagenesis experiments and by phosphorylation assays.

MATERIALS AND METHODS

PLASMID DNAs

The expression vectors used for stable transfection of Ltk$^-$ cells were pMV7, pMV7- $\alpha_o$wt, pMV7- α_oQL, pMV7- α_{i1}wt, pMV7- α_{i1}QL, kindly provided by Dr. Y. Wong. Transient transfections were performed with the following vectors: pcDNAI (Invitrogen); pREP8 and p163REH-PLCβ3 kindly provided by Dr Weber Gunther (Dept. of Molecular Medicine, Karolinska Institutet, Sweden), pcDNAI-Gα_q, pcDNAI-$\alpha_o$wt, pcDNAI-α_oQL, pcDNAI-β1, pcDNAI-β2 and pcDNAI-γ2 gently provided by B. Yung (The Hong Kong University of Science and Technology, HKUST); prk5 and prk5-α1AR gently provided by Dr. Susanna Cotecchia (Institut de Pharmacologie et de Toxicologie de Lausanne, Switzerland) and pcDNAI-PLC β4a, pcDNAI-PLC β4c which were reconstructed in our laboratory as described below.

These plasmid DNAs were all amplified by MC1061 strain of *E.coli.*

STABLE TRANSFECTION OF Ltk$^-$ CELLS

Ltk$^-$ cells (mouse fibroblasts) were transfected with pMV7, pMV7-α_o WT, pMV7-α_o QL, and pMV7-α_{i1} WT by the $CaPO_4$ method (Graham and van der Eb, 1973) and were selected by growth in the presence of 400 μg ml^{-1} G418. Individual clones were generated from the infected G418-resistant pools by limiting dilution. Uninfected cells, G418-selected pools and clonal

lines were mantained in colture in Dulbecco's modified medium supplemented with 10% fetal calf serum in 5% CO_2.

PLASMID DNA PREPARATION

A single colony of the bacterial strain carrying the plasmid was picked up from a freshly streaked plate containing 40 mg/ml ampicillin and inoculated in 5 ml Luria Broth (LB) containing 40 mg/ml ampicillin. The starter culture was incubated for 6h at 37 °C with vigorous shaking (300 rpm) and then diluted 1 : 500 into selective LB medium (500 ml). The culture was grown at 37 °C for 16 h with vigorous shaking till reaching an O.D. (600 nm = 2) corresponding to a cell density of 2×10^9 cells/ml.

Bacterial cells were harvested by centrifugation at 6000 x g for 15' at 4 °C. The pellet was resuspended in 38 ml resuspension buffer (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA) containing 100 μg/ml RNAse. Then 40 ml of lysis buffer (0.2M NaOH, 1% SDS) were added; the suspension was gently mixed and incubated for 10' at room temperature. Finally, 20 ml of chilled neutralization buffer (3 M K-acetate, 11.4% acetic acid) were added, mixed and incubated for 15' at 4 °C. The suspension was then centrifuged at 11500 x g for 30' at 4° and the supernatant was centrifuged again for 15' and filtered in another tube. To precipitate DNA and partially solubilize RNA 60ml of isopropanol were added and incubated for 15' at room temperature. The solution was centrifuged at 11500 x g at 4° for 15'. The supernatant was

decanted and the pellet resuspended in TE (10 mM Tris pH8, 1 mM EDTA) ; 740 μg/ml of ethidium bromide and 1g/ml CsCl were added. The suspension was centrifuged at 2500 x g for 15' at 4 °C to precipitate RNA and protein residues. The supernatant was filtered in Beckman Quick-Seal tubes (362181) and centrifuged in Beckman ultracentrifuge (TiV65 rotor) at 275000 x g for 16 h at 20 °C.

Two bands of DNA were visible in ordinary light (nicked circular plasmid DNA and closed circular plasmid DNA) and were collected into a 10ml siringe. Ethidium bromide was removed by adding 1 volume of isobutanol saturated with water, mixing and removing the upper organic phase. The extraction was repeated six to eight times until the pink color disappeared from both the phases. The aqueous phase was transferred into a Corex tube, diluted with 3 volumes of water (to remove CsCl) and 0.1 volume of Na-Acetate 3M pH5 and 2 volumes of ethanol 98% were added to precipitate DNA. The solution was incubated for 15' at 4° and then centrifuged at 16000xg for 10' at 4 °C. The supernatant was discarded and the pellet was resuspended in 400 ml TE and stored at -20°.

TRANSIENT TRANSFECTION OF COS7 CELLS

COS7 cells were mantained in Dulbecco's modified medium supplemented with 10% fetal calf serum in 5% CO_2 and were transfected by the DEAE-dextran method (Cullen B.R., 1987). Cells were seeded at $2x10^6$ cells/60 mm

dish and transfected 24 h later by incubation with 4ml of transfection mixture (15μg of plasmid DNA in DMEM/10% FCS containing 0.1 mM cloroquine and 0.4 mg/ml DEAE-dextran) for 4 hr. Cells were shocked with phosphate-buffer saline (0.13 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4) containing 10 % dimethyl sulfoxide for 2 min, then washed once with phosphate-buffer saline and mantained in growth media for 24 h.

MEASUREMENT OF INOSITOL PHOSPHATE (IP) ACCUMULATION

For experiments with Ltk$^-$ clones, cells were seeded at 2 x 10^5 cells/well in 12-well plates and 24 h later were incubated with myo [H ^{3}] inositol (4 μCi/ml) for 48 h. When requested, Na-butyrate was added to a final concentration of 10 mM for 36 h and pertussis toxin (PTX) was added to a final concentration of 0.1 μg ml^{-1} for 16 h. The cells were washed with 2 ml/well of assay medium (10 mM Hepes-buffered Dulbecco's modified Eagle's medium without serum and antibiotics) and incubated in the same medium at 37 °C for 1 h. Then cells were preincubated for 10 min at 37 °C with 1 ml of assay medium containing 20 mM LiCl and stimulated or not for 1 h at 37 °C with 100 μM ATP or LPA. After incubation, the medium was aspirated and cells were lysed by addition of 0.75 ml of ice-cold 20 mM formic acid. After 30 min, plates were centrifuged at 1000 x g for 10 min at 4 °C and the supernatant fractions were loaded onto Dowex 1x8 columns,

followed by immediate addition of 3 ml of 0.2% ammonium hydroxide ([^{3}H]inositol fraction). The columns were then washed with 10 ml of 40 mM ammonium formate/0.1 M formic acid followed by 4 ml of 2 M ammonium formate/ 0.1 M formic acid ([^{3}H]IP fraction). Combined radioactivity in these two fractions correlated directly with the number of the cells in each well. Accordingly, data are presented as the quotient of [^{3}H]IP divided by [^{3}H] inositol plus [^{3}H] IP.

For experiments with COS7 cells, 24 h after transfection each 60-mm dish of cells was trypsinized and resuspended in 8ml growth medium. This suspension was plated in 24-well plates (1 ml/well) and incubated with myo[^{3}H]inositol (5 μCi/ml). 24 h later the cells were washed twice with 2 ml of assay medium (10 mM Hepes-buffered Dulbecco's modified Eagle's medium without serum and antibiotics) and incubated at 37 °C for 15 min with 400 ml of assay medium containing 10 mM LiCl. After addition of 400 ml of the same medium with or without 2 μM phenylephrine, cells were incubated for 30 min at 37°. The medium was then aspirated and cells were lysed by addition of 0.75 ml of ice-cold 20 mM formic acid. Inositol phosphate were separated as described above.

MEASUREMENT OF cAMP

For these experiments, confluent cells in 24-well plates were washed twice with 1 ml of assay medium (10mM Hepes-buffered Dulbecco's modified Eagle's medium without serum and antibiotics), and incubated in 500 µl of the same medium containing 1 mM IBMX for 10 min at 37 °C in the absence or presence of 20 µM forskolin. Reactions were terminated by aspiration of the medium and addition of 500 µl boiling acetate buffer (0.5 mM sodium acetate pH 6.2). After 20 min, the plates were centrifuged at 1000 x g for 10 min at 4 °C. cAMP was determined by radioimmunoassay using a commercial kit (Du Pont New England Nuclear). The DNA content of the wells was determined as described (Valente et al., 1983).

REVERSE TRANSCRIPTION-PCR ANALYSIS

To detect expression of the various PLC β isozymes, total RNA was prepared from Ltk$^-$ cells rat brain and HL60 cells using the guanidinium thiocyanate phenol-based single-step method (P.Chomczynski, N.Sacchi, 1987). cDNA was synthesized by using the GeneAmp RNA PCR Kit (Perkin Elmer) according to the protocol suggested. For PLC β1, the reaction proceeded for 35 cycles of 94 °C for 1', 60 °C for 45'', and 72 °C for 2'. The two primers used were: PB1F (5'-GACTCATCCAACTACATGCC-3') and

PB1R (5'-GTCTGCGCCTCTACTTCTGT-3'). For PLC β2, the reaction proceeded for 35 cycles of 94 °C for 1', 60 °C for 45'', and 72 °C for 2'. The two primers used were: P2/3F (5'-GACTCCTCCAACTACATGCC-3') and PB2R (5'-CTGGACTCGCCAGTGGGAA-3'). For PLC β3, the reaction proceeded for 35 cycles of 94 °C for 1', 60 °C for 1', and 72 °C for 2'. The two primers used were: PB2/3F (previously described) and PB3R (5'-AAGCTGGGTGTTTTCCTCCT-3'). For PLC β4, the reaction procedeed for 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The two primers used were: PB4F (5'-AGATTGTGGCGCAGCACACA-3') and PB4R (3'-CTGTTGCTGGTCTGCGGTC-5').

To investigate the pattern of expression of PLC β4a and PLC β4c, total RNA was prepared from adult rat tissues as described above.

For detection of PLC β4a and PLC β4c, the reaction procedeed for 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The two primers used were: PB4F and PB4R.

For detection of PLC β4b and PLC β4c, the reaction proceeded for 30 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. The two primers used were: PB4Co (5'-AGATTCAGACCCTGACATC-3') and PB4RES (5'-CACTGCATGACAGGATTTCAA-3'). Oligonucleotides were synthesized by Primm (DiBit, Milano). All amplified products were analyzed in a 1% agarose gel stained with ethidium bromide. The DNA molecular weight marker used to discriminate the bands was *fX174*, New England Biolabs cod. N. N3026S.

cDNA sequencing

cDNAs amplified in RT-PCR were eluted from the agarose gel and sequenced by the Sequenase PCR Product Sequencing kit (US70170, Amersham), using primers PB4F and PB4R and PB4F2 (5'-AGAGACTGGCAATGAAGCA-3').

Southern Blot

1 % agarose gels containing RT-PCR products were fixed for 20' in NaOH 0.4 N and blotted for capillarity in SSC10X (1.5 M NaCl, 0.15 M Na-cytrate) overnight on a nylon membrane (Biodyne, B. PALL) . The membrane was washed in SSC2X, fixed for 2 h at 80 °C and prehybridized for 6 h at 50 °C with 25ml hybridization buffer (0.125M Na_2HPO_4, 8% H_3PO_4, 1 mM EDTA pH 8, 0.25M NaCl, 7% SDS, 10% PEG8000, 1% BSA) containing 0.2 mg/ml predenaturated salmon sperm. It was then hybridized for 16 h at 50 °C with 15ml of the same hybridization buffer containing 0.4 mg/ml salmon sperm and 15 x 10^6 cpm of [^{32}P]αdCTP labelled probe 5'-CTTTTGAAATCCTGTCATGCAGTGTCCCAAACACAAG-3'. This probe was synthetized by Primm, labelled by Random Priming method according to

the standard protocol of DecaPrime II, Celbio Ambion and purified by G50 columns, Pharmacia. The membrane was washed with SSC2X/0.1%SDS for 30' two times and exposed for 15' at –80 °C to Kodak (X-OMAT AR) film.

CLONING OF PLC β4a AND PLC β4c

Isolation of cDNA

Two DNA probes were used to screen a rat brain λZapII cDNA library in order to identify clones containing sequences corresponding to PLCβ4 a and PLCβ4 c variants. Two clones were identified by PCR analysis on phages which corresponded to the two proteins. Both the clones contained the 3' terminal of the sequence.

The two clones (11A, 3,5Kb long, for PLC β4a and 81A, 3,4Kb long, for PLC β4c) were plaque purified and subcloned into Bluescript vectors (respectively named 3' SK β4a and 3' SK β4c) by in vivo excision with ExAssist helper phage. To characterize the phagmides rescued, a restriction analysis and a sequence analysis were performed (radiolabeled reaction with Sequenase Kit Version 2 and sequence detection by vertical gel electrophoresis).

Isolation of full-length cDNA encoding PLC β4a and PLC β4c

In order to obtain the 5' sequence of PLC β4 cDNA, total RNA was prepared from adult Sprague Dawley rat brain tissue using the guanidinium thiocyanate phenol based method (Chomczynski and Sacchi, 1987). RNA was reverse transcribed with a specific antisense primer (oligo B: 5'-CGGCTCATTTGTCGCTTATTG-3') encompassing the 5' terminal of both the clones 11A and 81A. The reaction was performed in a 20 µl mixture containing 50 mM Tris-HCl pH 8.3, 3 mM $MgCl_2$, 75 mM KCl, 0.5 mM dNTPs, 10 mM dithiothreitol, 40 units of RNAse inhibitors, 10 µM reverse primer, 200U Moloney Murine Leukemia Virus reverse transcriptase. 3 µg RNA were heated in 7µl of water at 65 °C for 15' and were added to the buffer and incubated at 37 °C for 1 h. The reaction was stopped by 10 min on ice. 1µl of the reaction mixture was used for PCR amplification. PCR was carried out in a 50 µl reaction mixture containing 1 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2mM dNTPs, 50 picomoles of each primer, 1U of PFU polymerase. The reaction proceeded according to a touch down protocol: denaturation of 1' at 94 °C; 30 cycles of 94°C for 45'', 62 ° -60 °-58 °C for 45'', 72 °C for 8'; a final extension of 72 °C for 5'. The two primers used were the sense primer PB4F3 (5'-TTCGATCAGCTAGACCTCCC-3') and the antisense primer oligoB. The amplified PCR products were analyzed on a 1% agarose gel stained with ethidium bromide. The 5' terminal fragment obtained from RT-PCR was

cloned into the EcoRV site of a pBluescript SK plasmid. The resulting plasmid was named 5' SK β4.

Construction of Mammalian Expression Vectors

The mammalian expression vectors for PLC β4a and PLC β4c were constructed by inserting the BstuI-HindIII fragment of 5'SK β4, the BstuI-SphI fragment of 3' SK β4a and 3' SK β4c respectively, into the HindIII-SphI site of pcDNAI. All DNA sequences were verified by sequencing (fluorescent dye terminator method and capillary electrophoresis, 3700 Perkin-Elmer).

ANALYSIS OF PROTEIN EXPRESSION

Analysis of the expression of G α_o in Ltk$^-$ clones

Ltk$^-$ clones coltured in 10 mm dishes were detached by 0.5 mM PBS/EDTA and centrifuged. The pellet was frozen at –80 °C for at least 3 hr and then homogenized with a buffer (5 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, 1 mM βmercaptoethanol) containing 3 mM benzamidine, 1 mM phenylmethylsulphonylfluoride, 2 μg/ml Soy bean

Trypsin inhibitor, 2.5 μg/ml chymostatin, 2.5 μg/ml leupeptin, 2.5 μg/ml antipain and 2.5 μg/ml pepstatin. Membranes were separated by centrifugation at 100000 x g at 4° for 60' in a Beckman TL-100s ultracentrifuge, resuspended in a buffer containing 20 mM Tris-HCl pH 8, 2 mM MgCl2, 1 mM EDTA, 0.007% βmercaptoethanol, 10% glycerol and stored at –20 °C. Proteins were measured by BCA (Bicinchoninic Acid)(Protein Assay Reagent, Pierce). A volume corresponding to 100 μg of proteins was drawn from each sample, mixed (30 : 21) with solubilization mix (4 % SDS, 0.8 M saccharose, 8% βmercaptoethanol, 0.008% bromophenol blue, 48 mM Tris pH6.8), boiled for 5', centrifuged briefly and fractionated by 10% polyacrylamide gel. Electrophoretic migration proceeded at 200 Volts for 5-6 h in buffer containing 50 mM Tris, 384 mM Glycine, 0.1% SDS. The proteins were then electroblotted onto a nitrocellulose membrane (Schleicher and Schuell) at 4 °C overnight at 250 mA in 25 mM Tris, 192 mM glycine and 20% methanol. The membrane was preincubated for 1hr at room temperature in Blotto (50 mM Tris pH7.4, 150 mM NaCl, 0.01 % Na-azide, 0.05 % Tween 20, 5 % non fat dry milk). A rabbit polyclonal antibody, raised by our laboratory against a peptide corresponding to the C-terminal ten aminoacids of G α_o, was diluted 1:200 in Blotto and incubated with the membrane at 4° overnight. The filter was then washed with Blotto two times for 15' and three times for 5'. Binding of the antibody was detected by incubating the filter with [^{125}I] protein A (0,4 μCi/ml) diluted

in Blotto 1 h at room temperature. Then, the filter was washed with Blotto three times for 10 min and with TBS (50 mM Tris pH 7.4, 150 mM NaCl) six times for 3 min. Filter was exposed to autoradiographic films (Hyperfilm).

Analysis of the expression of G α_q

Membrane from COS7 cells transfected in 10mm dishes were obtained as described previously for Ltk$^-$ clones. 30 μg of proteins mixed (30 : 21) with a solubilization mix (4 % SDS, 0.8 M saccharose, 8 % βmercaptoethanol, 0.008 % bromophenol blue, 48 mM Tris pH6.8) were boiled for 5', centrifuged briefly and fractionated by 10 % polyacrylamide gel. Electrophoretic migration proceeded at 200 Volts for 5-6 hr in buffer containing 50 mM Tris, 384 mM glycine, 0.1% SDS. The proteins were then electroblotted onto a nitrocellulose membrane (Schleicher and Schuell) at 4 °C overnight at 250 mA in 25 mM Tris, 192mM glycine and 20% methanol. The membrane was preincubated for 1hr at room temperature in Blotto. Anti-G αq rabbit polyclonal antibodies (Santa Cruz) diluted in Blotto (0.2 μg/ml) were incubated with the membrane at 4° overnight. The filter was then washed with Blotto two times for 15' and three times for 5'. Binding of the antibody was detected by incubating the filter with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit antibody in Blotto for 1hr at room temperature. The filter was washed with Blotto two times for 10', and three times for 5' and with TBS one time for 5'. Immunolabeling was

detected by ECL (enhanced chemioluminescence) system (Amersham) and visualized by exposure of the filter to Amersham Hyperfilm MP films.

Analysis of PLC β4a and PLC β4c expression

Membrane preparation, separation of proteins (200 μg) on 6 % polyacrylamide gels and immunoblotting were performed as described for detection of G αq. Rabbit polyclonal antibodies against PLC β4a (Santa Cruz) were used at a concentration of 0.2 μg/ml in Blotto. The rabbit polyclonal antibody was generated against the residues GSRDGPQTSNSSMKL of the C-terminal tail of PLC β4c and coupled to KLH (keyhole limpet hemocyanin) by Eurogentec; then it was affinity purified in our laboratory on Sepharose protein G columns (Pharmacia) or on peptide conjugated Tresyl Activated Agarose columns (Pierce) and diluted at 2 μg/ml in Blotto.

Intracellular localization of the two PLC β4 variants

Tissues (~ 500mg) and cell lines were homogenized by pottering with homogenizing buffer (5 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, 1 mM mercaptoethanol) containing 3 mM benzamidine, 1 mM phenylmethylsulphonylfluoride, 2 μg/ml S. Trypsin inhibitor and 2.5μg/ml

chymostatin, 2.5 μg/ml leupeptin, 2.5 μg/ml antipain, 2.5 μg/ml pepstatin. The suspension was centrifuged at 100000 x g at 4 °C for 60 min in a Beckman TL-100s ultracentrifuge. The supernatant was separated from the pellet. This was resuspended in freezing buffer (20 mM Tris-HCl pH8, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM βmercaptoethanol, 10% glycerol). All samples were stored at –20 °C. Proteins were measured by BCA, separated by 6% SDS-polyacrylamide gel electrophoresis and detected as described above.

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